INFECTION OF CHICKEN ERYTHROCYTES WITH INFLUENZA AND OTHER VIRUSES

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Summary

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The first section investigates the production of viral polypeptides and infectious progeny virions from avian erythrocytes infected with fowl plague virus, Newcastle disease virus and Semliki Forest virus.

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My special thanks go to my parents for their support over many years; to all the other inmates of the Fowl Plague laboratory for generating a cultured, academic environment and to Bram Stoaker for providing much of the inspiration for this work.

Declaration

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which this is a record has been done by myself, and all sources of information have been specifically acknowledged by means of references.

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Abbreviations

AMD	actinomycin D
BHK	baby hamster kidney cells
BSA	bovine serum albumin
CEF	chick embryo fibroblasts
CHO	Chinese hamster ovary cells
CM	cycloheximide
cpm	counts per minute
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DNase	deoxyribonuclease
EB	erythroblast
EPE	early-polychromatic erythrocyte
FANA	2-deoxy-2,3,dehydro-N-trifluor-acetylneuraminic acid
FP/Dutch	fowl plague strain Dutch
FP/R	fowl plague strain Rostock
FP/Weibridge	fowl plague strain Weybridge
FP/V	fowl plague virus - strain unspecified
GMEM	Glasgow modified Eagle's medium
HA	haemagglutinin
HAU	haemagglutinin units
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
LPE	late-polychromatic erythrocyte

M	matrix protein
MDBK	Madin-Darby bovine kidney cells
MDCK	Madin-Darby canine kidney cells
ME	mature erythrocyte
m.o.i.	multiplicity of infection
MPE	mid-polychromatic erythrocyte
NA	neuraminidase
NDV	Newcastle disease virus
NP	nucleoprotein
NP40	Nonidet P40
NS	non-structural
O.D.	optical density
P	polymerase protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
p.f.u.	plaque forming unit
poly A	poly-adenosine
PPO	2-5 diphenyloxzole
RNA	ribonucleic acid
RNase	ribonuclease
cRNA	virus RNA complementary tothe influenza genome
hnRNA	heterogenous RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
vRNA	viral genome

RNP	ribonucleoprotein
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
TCA	trichloracetic acid
TCA↓	trichloracetic acid precipitable
tris	Tris (hydroxymethyl) aminomethane
ts	temperature sensitive

GENERAL INTRODUCTION

1. Preamble

Influenza has been referred to as the 'last of the great plagues', a fitting description for a disease which so frequently rages in pandemic proportions. The term influenza first appeared in 1743 and is a corruption of an Italian name, 'the influence', a reference to the belief that the disease was caused by the influence of the planets. However, the disease causing agent is not the relative position of the heavenly bodies but a virus. This was first isolated from pigs in 1931 (Shope, 1931) and then two years later from humans (Smith et al., 1933). Since then the virus has been extensively studied, yielding much information about its structure, its replication and its immunology. Yet despite some progress in vaccine preparation, perhaps the only value of these advances to influenza sufferers are that they illustrate the difficulties of curtailing the disease.

The occurence of influenza follows a pattern in which there is a major epidemic, often of pandemic proportions, followed by a number of years of much lower incidence. This pattern can be correlated with changes in the viral surface antigens, haemagglutinin (HA) and neuraminidase (NA). These antigens undergo radical changes, antigenic shift, or relatively minor but more frequent changes, antigenic drift. Radical changes in surface antigens often lead to pandemics. For example, in 1957 the Asian virus bearing surface antigens previously unrecognised in man (designated H2 N2) replaced viruses of

To be Corrected.

the H1 N1 antigen subtype which had been prevalent since 1946. (The 1971 recommendations of an expert group of the World Health Organisation regarded H1 as a distinct subtype which in 1946 replaced the H0 subtype. H0 had been prevalent since 1932. However, H0 and H1 appear to be antigenically related (Schild, 1970; Baker et al., 1973; Schild et al., 1980) and therefore it has been proposed that they be reclassified as belonging to the same subtype (Schild et al., 1980)). There followed the largest influenza pandemic recorded since the 1930's. After 1957 the incidence of the disease dropped markedly and remained at a relatively low, but constant, level until 1968. During this time influenza virus isolates were all of the H2 N2 subtype, having undergone only minor changes from the original. However, in 1968 a major shift was detected in the HA antigen. This new subtype, Hong Kong (H3 N2), was responsible for an epidemic in America in 1968 and for an epidemic in Britain in 1969.

There are three principal suggestions for the origin of new subtypes. The first is that there is some animal acting as a reservoir for new viruses. That this may be feasible is shown by the case of Fort Dix in 1976 when a swine influenza strain was transferred to man. However, this transfer was epidemically abortive. The second is that new viruses might occur through mutation of existing human strains. Such a hypothesis would demand multiple changes in the genes coding

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for the surface antigens. For instance, amino acid sequence analysis has shown that there are major differences in the HA polypeptide between the Hong Kong influenza strain and the 1956 H2 N2 strain which preceded it (Laver and Webster, 1972). These authors concluded that mutation alone was unlikely to generate such major differences.

The third possibility and the one which has the most experimental support is that new subtypes arise from genetic interaction of existing human strains with influenza viruses of other mammals or birds. Many mammals and birds are naturally infected with influenza A viruses (Pereira et al., 1967). Mixed infections of cells in tissue culture (Laver and Kilbourne, 1966; Easterday et al., 1969; Webster, 1970) and of animals (Webster et al., 1973) with more than one type of influenza virus results in the formation of recombinant virus particles at a high frequency. The ease with which such recombinants form is due to the segmented nature of the influenza virus genome (see below) which readily allows the reassortment of genes. This high frequency recombination occurs under conditions resembling those of natural transmission (Webster et al., 1973). Furthermore, there is some evidence that the 1968, H3 N2 subtype arose by recombination of the Asian (H2 N2) virus and a non-human strain related to A/Duck/Ukraine/63 (H av 7 Neq 2) and A/Equine/Miami/1/63 (H eq 2 Neq 2) viruses (Laver and Webster, 1973).

The mechanism of antigenic drift involves selection pressure of neutralizing antibody on mutations arising at the antigenic sites. After a major epidemic, herd immunity to the causative strain would be high. Therefore, variants arising through antigenic drift would have a growth advantage. This phenomenon can be duplicated in the laboratory by growing virus in the presence of limiting dilutions of specific antibody (Laver and Webster, 1968).

With careful virological surveillance by the World Health Organisation since the late 1940's it has become possible to observe any re-emergence of HA or NA antigen, when herd immunity no longer excludes it from the human population. Such a case was observed in an epidemic of 1977-78 when a strain indistinguishable immunologically from the classical H1 N1 subtype appeared (reviewed by Pereira, 1979). How and where this strain had been preserved for twenty years remains a mystery.

These then are some of the properties by which influenza has earned the title 'last of the great plagues'. The high incidence of the disease (30-80% of the human population in a pandemic year) with its often fatal consequences for the old and sick justify much of the research effort into influenza. However, studies into how a virus, with its relatively small amount of genetic information, interacts

with and controls its host cell may provide insight, not only into a particular disease but also into the mysterious array of organisational and regulatory processes that occur within eukaryotic cells. The interaction of influenza with its host cell is particularly interesting as the virus has an unusual and absolute requirement for a functional host cell nucleus (see below). It was on the theme of the interaction of influenza with its host cell that the work presented in this thesis was initiated. Earlier work from this laboratory had indicated that heterokaryons formed between avian erythrocytes and "enucleated" B.H.K. cells (Kelly and Dimmock, 1974; Minor and Dimmock, 1976) could permit the synthesis of some but not all influenza proteins. This had suggested that the avian erythrocyte nucleus, which is metabolically dormant by comparison with nuclei from dividing cells (see below), could provide some but not all the requirements for influenza replication (Kelly and Dimmock, 1974; Minor and Dimmock, 1976).

A further, quite separate, reason for attempting to study viral replication in erythrocytes stems from reports that during and after infection, viruses of many different types, are found bound to or within these cells (Powick, 1937; Traub, 1938; Schwartzman, 1944; Epstein *et al.*, 1951; Hamre *et al.*, 1956; Mims, 1956, 1964; Overman, 1958; Sirbu *et al.*, 1964; Bernard *et al.*, 1968; Luedke, 1970;

Nandi and Haslam, 1971; Reilly and Schloss, 1971; Oshiro *et al.*, 1972; Emmons *et al.*, 1972). Should erythrocytes permit viral replication, i.e. that the association of the cells with viruses is not entirely passive, then this might have some relevance to the disease process.

Section 1 of this thesis is concerned with the production of viral polypeptides and infectious progeny virions from avian erythrocytes infected with Fowl Plague Virus, Newcastle Disease Virus and Semliki Forest Virus. In Section 2 a closer examination is made of viral polypeptide synthesis in avian erythrocytes following infection with avian and/or human influenza strains. In Section 3 the distribution of newly synthesized influenza viral polypeptides between the erythrocyte nucleus and cytoplasm is investigated while Sections 4 and 5 are concerned with the relationship between virus infection and the state of differentiation of erythrocytes.

2. Structural components of influenza viruses

The influenza viruses are divided into three types, A, B and C according to the serologic relatedness of their nucleocapsid and matrix proteins. The three types resemble each other in morphology (Flewett and Apostolov, 1967) with the A and B types having resemblances in the arrangement of their surface projections (Apostolov *et al.*, 1970; Compans *et al.*, 1977). All three types contain a receptor destroying enzyme. However, type C viruses differ from the A and B types in that their receptor destroying enzyme is not an α-neuraminidase (Kendal, 1975). Influenza A viruses have been isolated from a variety of animals including man, whereas B and C viruses have, so far, been found only in man.

a) Morphology of the influenza A viruses

Influenza viruses have been observed in spherical or in filamentous forms. Both these forms have diameters of between 80-120 nm but the filamentous forms are greatly elongated, being at least 4 μm in length. It is the filamentous forms which predominate in newly isolated virus strains (Choppin *et al.*, 1960) but with serial passage in culture, particularly in the chick embryo, conversion from filamentous to spherical morphology is observed (Choppin *et al.*, 1960). This is the probable reason why most laboratory grown stocks of influenza A viruses are found to be spherical.

The virus has been shown to consist of an outer lipid membrane, derived from the host cell (Kates et al., 1961; Klenk and Choppin, 1969, 1970) from which projects a series of spikes. These spikes are about 10 nm in length and there are between 700 and 900 per spherical virus particle (Hoyle et al., 1961; Wrigley, 1979). The arrangement of the spikes is roughly similar to that of a honeycomb with each one having approximately six neighbours. The spikes themselves consist of the virus polypeptides, haemagglutinin (HA) and neuraminidase (NA). HA is the major surface antigen, comprising approximately 25% of the virion protein (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971). It is the product of a single gene but is cleaved during normal maturation into a heavy and a light chain (Lazarowitz et al., 1971, 1973; Stanley et al., 1973). These are designated HA1 and HA2 respectively. HA1 and HA2 are bound by disulphide bridges into a single subunit. Studies with cross-linking reagents has shown that the rod-shaped HA spikes are trimers of such units (Wiley et al., 1977). The other type of spike, the NA, appears to be a mushroom-shaped tetramer (Wrigley et al., 1973; Wrigley, 1979). Both the HA and NA have hydrophobic ends with which they are embedded in the lipid bilayer. The depth to which these proteins are embedded is unknown. However, nucleotide sequence analysis of Fowl Plague Virus haemagglutinin gene (Porter et al., 1979) has shown that the hydrophobic region

of the protein may not be directly at the C' terminus since in between there is coding capacity for 11 hydrophilic amino acids. If this hydrophilic portion is retained in the functional protein (this is at present unknown since the overall hydrophobicity of the C^1 terminal region has prevented determination of its amino acid sequence (Ward and Dapheide, 1979; Waterfield et al., 1979)) it would imply that HA spans the membrane.

Inside the lipid bilayer is a shell of protein some 6 nm thick (Apostolov and Flewett, 1969). This shell is thought, by elimination rather than experiment, to be composed of a single protein having a MW of 25,000. Despite being the smallest of the virion structural polypeptides this protein, termed the matrix or M protein, is the most abundant. It accounts for between 33 and 46% of the total virion protein (Compans et al., 1970; Schulze, 1970; Skehel and Schild 1971).

The structure and function(s) of M protein are not known with any certainty (see below). However, the viral membrane is considerably more rigid than that of the host plasma membrane (Landsberger and Compans, 1976). Speculation has arisen that M protein is involved in generating this greater structural rigidity (Landsberger et al., 1978). For instance, Lenard et al. (1976) observed that M protein

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penetrates the membrane. Support for this comes from Reginster et al. (1976) who have found that about half of the M protein is susceptible to digestion by proteolytic treatment of intact virions.

Within the M protein shell are ribonucleoprotein structures. These are composed of one major protein, the nucleoprotein (NP), which coats the viral RNA and three high molecular weight proteins (P1, P2 and P3) (Inglis et al., 1976; Lamb and Choppin, 1976; Ritchey et al., 1977; Stephenson et al., 1977). Although it is almost certain that influenza RNA is associated in some regular manner with molecules of nucleoprotein, the structure of the ribonucleoprotein complex is a matter of some debate. The electron microscope occasionally shows what appear to be cylindrically coiled filaments of about 7 nm thickness, but they are seen only in extensively degraded particles where stain has been allowed to penetrate. Such coils are extremely variable in respect of diameter and number of turns but are remarkable for their uniformity of coiling and may well reflect some reorganization due to particle disruption (Schulze, 1972). Furthermore, it is known that RNA of influenza virus is not a continuous filament but that it is segmented (Davies and Barry, 1966; Duesberg, 1968; Pons and Hirst, 1968; Skehel, 1971). Centrifugation of the ribonucleoprotein (RNP) structure in velocity gradients, following disruption of the

virus particle, show them to be of different size classes (Duesberg, 1969; Pons, 1971; Compans et al., 1972). Although it has not been shown conclusively it is widely believed that each RNA, from a disrupted virus particle, is in the form of a separate RNP structure. It is not known whether these structures remain separate in the intact particle or whether they are linked to form some specific complex.

b) Influenza A RNA

Analysis of the nucleic acid of influenza A viruses revealed the presence of single-stranded RNA (Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956). As already indicated this RNA is composed of several individual units, i.e. it is segmented. This segmented nature was originally suggested from recombination studies (Burnet, 1956; Barry, 1961) a concept supported by analysis of the RNA on sucrose density gradients and polyacrylamide gel electrophoresis (PAGE) (Davies and Barry, 1966; Duesberg, 1968; Pons and Hirst, 1968; Skehel, 1971a). The resolution of single stranded RNA on PAGE was found to be improved by the addition of high concentrations of urea (Floyd et al., 1974). Using this technique the influenza genome can be resolved into eight segments (McGeoch et al., 1976; Palese and Schulman, 1976; Pons, 1976; Bean and Simpson, 1976). That these are unique was confirmed by oligonucleotide mapping (McGeoch et al..

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1976). In some strains however, two (Hay *et al.*, 1977) or more additional segments are found (Crumpton *et al.*, 1978; Nayak *et al.*, 1978).

RNA isolated from virions (virion RNA or vRNA) can be almost completely protected from nuclease digestion by hybridization with RNA isolated from infected cell polysomes (Etkind and Krug, 1975; Glass *et al.*, 1975). Furthermore, vRNA does not direct the synthesis of virus specific polypeptides in *in vitro* translation systems. These observations indicate that vRNA does not act as mRNA. Some limited support for this conclusion is provided by the observation that influenza vRNA does not contain a 7' methyl G capped 5' terminal or a 3' poly(A) tail. These are structures commonly found in mRNA species.

That the vRNA of influenza viruses does not act as a messenger and that the virus contains an RNA polymerase activity (Chow and Simpson, 1971; Simpson and Bean, 1975) results in influenza being classified as a negative strand virus (Baltimore, 1971).

3. Mapping of the influenza genome

The influenza genome specifies at least eight polypeptides (Inglis et al., 1976). There are three polymerase associated proteins (P1, P2, P3), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), membrane or matrix protein (M) and a non-structural polypeptide (NS1). The molecular weights of these polypeptides are shown in Table 1. In some studies further, smaller polypeptides have been observed (Skehel, 1972; Follet et al., 1974; Minor and Dimmock, 1975, 1977; Etkind and Krug, 1975). These polypeptides have a MWs of ~21,800, 19,000, 17,000 and 11,000 (Lamb and Choppin, 1978). Occasionally a fifth small polypeptide (MW ~8,000) has been observed in certain cell lines (Lamb and Choppin, 1978). By tryptic peptide analysis the polypeptides of MW ~21,800, 19,000 and 17,000 appear to be cleavage products of NS1 (Lamb and Choppin, 1978), but the polypeptide of MW 11,000 is unique (Lamb et al., 1978) and has been designated NS2.

Molecular weight correlations led to the first tentative assignments of viral polypeptides to their genes (Pons, 1976). Since then more sophisticated methods have been developed and the genetic maps of several influenza A and B strains have been obtained.

Table 1

Molecular weights of influenza virus proteins

Viral Protein	MW ($\times 10^3$)	Estimated number of molecules/virion[c]
P1[a]	95	15-14
P2[a]	87	17-16
P3[a]	85	17-16
HA[b]	75-80	
NP[b]	55-65	1,000
NA[b]	55-70	200
M[b]	21-27	2,400
NS1[b]	23-25	
HA1[a]	44	1,018
HA2[a]	26	1,141

a. Values for fowl plague virus (Inglis et al., 1976; McGeoch et al., 1976; Rohde et al., 1977).

b. Molecular weights representing average values determined in different laboratories with different influenza virus strains (Choppin and Compans, 1975; Pons, 1976; Ritchey et al., 1976; McGeoch et al., 1976; Inglis et al., 1976; Rohde et al., 1977).

c. From Inglis et al., 1976.

One of these methods relies on generating recombinants by infecting cells with a temperature sensitive (ts) mutant virus of known defect in function and a 'rescuing' prototype influenza strain. As selection of recombinants is carried out at the non-permissive temperature they do not contain the defective gene(s) of the ts mutant. This gene(s) is replaced by the wild-type gene(s) from the 'rescuing' virus. This recombinant virus is used to infect cells from which virus specified complementary RNA (cRNA) is extracted. Hybridization of this RNA with vRNA of the ts mutant parent results in protection from S1 nuclease digestion, of those RNA segments of the recombinant that are derived from the ts mutant. Likewise, hybridization with vRNA from the other parent, followed by S1 nuclease digestion will reveal which RNA segments of the recombinant virus are derived from the rescuing strain. In this way a defect in function can be correlated with a specific RNA segment. These assignments can be extended to the corresponding proteins by comparing tryptic peptide fingerprints of each of the gene products of the two parent strains with fingerprints of the gene products of the recombinant (Schottlssek *et al.*, 1976; Rohde *et al.*, 1977; Harms *et al.*, 1978).

A second method takes advantage of the observation that the electrophoretic migration rates of the RNA segments and of the virus-specific protein are different for different

influenza strains (Palese and Schulman, 1976 (a); Ritchey et al., 1977). These differences may be due to slight variations in MW and/or to variations in secondary structure (Palese and Schulman, 1976 (b)).

Recombinant viruses are prepared by co-infection of cells with two different influenza strains. The electrophoretic migration characteristics of the RNAs and protein of the recombinants are then compared with those of the parent strains.(Ritchey et al., 1976; Palese et al., 1977 (a); Ritchey et al., 1977). If for example a recombinant contains all the RNAs from one parent except segment 8 and comparative analysis of the polypeptides of that recombinant show that NS1 is the only polypeptide different from that parent then segment 8 must code for polypeptide NS1.

A third method utilizes the phenomenon of hybrid arrest. Complementary RNA (cRNA) extracted from infected cells can be translated in cell-free protein-synthesizing system to give virus specific polypeptides (Kingsbury and Webster, 1973; Etkind and Krug, 1975). If cRNA is hybridized to vRNA prior to translation then no polypeptides are produced since double-stranded RNA is not translatable. So hybridization of a specific segment of vRNA to cRNA will only cause the loss of one of the viral polypeptides on translation. For example, if vRNA segment 4 causes the loss of

HA when hybridized to cRNA then segment 4 encodes HA (Inglis, 1977; Etkind et al., 1977).

The development of these methods has allowed the assignments of the eight RNA segments of influenza A viruses to the corresponding gene products. The assignments for the fowl plague strain Rostock and for the WSN strain are shown in Table 2, as can be seen there is some variation in the genetic maps of different influenza strains.

The assignment for NS2 is more interesting. Inglis et al. (1979) have identified a viral mRNA encoding NS2 in FP/R infected CEF cells. This mRNA hybridized to gene segment 8 which as shown in Table 2 also codes for NS1. From its MW gene segment 8 would appear to be too small to code for both polypeptides independently and it would seem likely that the NS1 and NS2 genes overlap. Furthermore, if, as the peptide maps suggest, the NS1 and NS2 polypeptides are completely unrelated, the genetic code of the shared nucleotide sequences would have to be read in two different frames.

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Table 2

Assignments of RNA segments to the gene products of Fowl Plague Rostock and WSN

Fowl Plague Virus[a] (Hav1 N1)		WSN Strain[b] (HON1)	
RNA Segment No	Corresponding Protein	RNA Segment No	Corresponding Protein
1	P2	1	P3
2	P1	2	P1
3	P3	3	P2
4	HA	4	HA
5	NP	5	NP
6	NA	6	NA
7	M	7	M
8	NS1	8	NS1

(a) Barry et al., 1978

(b) Reviewed by Scholtissek, 1978

4. Functions of Influenza Polypeptides

Analysis of ts mutants along with genetic mapping of influenza viruses has led to the elucidation of some of the functions of viral polypeptides.

a) P Proteins

Influenza virions contain an RNA-dependent RNA polymerase (Chow and Simpson, 1971; Penhoet et al., 1971; Skehel, 1971b), the active components of which were found to be viral RNA and the NP and P polypeptides (Bishop et al., 1972). A virus-induced RNA polymerase activity is also found in influenza virus-infected cells. The active components of this are the NP and P polypeptides (Calguiri and Compans, 1974). These early studies indicate that NP and P polypeptides are involved in viral RNA synthesis. The discovery that there were three P polypeptides raised questions as to their individual roles in viral RNA synthesis.

Experiments involving the influenza strain WSN indicated that proteins P1 and P3 were required for complementary RNA (cRNA) synthesis (Palese et al., 1977b), whereas P2 protein and NP were probably associated with the synthesis of virion RNA (Ritchey and Palese, 1977). In a study (Mowshowitz, 1978) on the requirement of primers (see below) in in vitro

translation systems by a ts mutant of WSN which had a defect in the P1 protein, it was suggested that P1 is required for initiation of cRNA synthesis while P3 functions in the elongation process.

Results from studies using ts mutants should be treated with some caution. For example, ts mutants defective in P1 and P3, of WSN and fowl plague have been isolated which lack vRNA synthesis as well and cRNA synthesis (Scholtissek et al., 1974; Scholtissek and Bowles, 1975; Scholtissek et al., 1976; Almond et al., 1978). Likewise, a ts mutant of fowl plague Rostock lacking in P2 has been found to be defective in both cRNA synthesis and vRNA synthesis (Scholtissek, 1978). Furthermore, some FP/R ts mutants, defective in P3 recombination group, have been shown to be lacking only in the transport of the polymerase complex from the nucleus to the cytoplasm (Scholtissek and Bowles, 1975). This indicates a degree of leakiness in the ts mutants or that certain viral products have more than one function, and that these functions can be affected independently by mutation (see review by Scholtissek, 1978).

b) Nucleoprotein (NP)

Besides a possible role in vRNA synthesis, NP forms the main structural component of viral RNPs (Hoyle et al., 1961; Pons et al., 1969; Krug, 1971). A ts mutant containing a

lesion in the NP gene has been found to synthesize normal yields of viral RNA and of other viral components but not to produce infectious progeny virus (Scholtissek and Bowles, 1975) possibly because of a failure to synthesize (functional?) RNPs.

c) Haemagglutinin (HA)

Haemagglutinin is a glycoprotein, containing the sugars, glucosamine, mannose, galactose and fucose. These sugars are arranged in two classes of carbohydrate chain. The first, or type I chain, contains all four sugars while the second, or type II chain, is smaller and contains only mannose and galactose (Schwarz et al., 1977; Nakamura and Compans, 1978). The molar ratio of these sugars and the proportion of type I to type II carbohydrate chain is determined by both the virus strain and the host cell (Nakamura and Compans, 1979; Schwarz et al., 1977).

Under certain conditions, for example treatment of infected cells with 2-deoxyglucose or glucosamine, the addition of sugar molecules to haemagglutinin is inhibited (Klenk et al., 1972). The resulting unglycosylated forms of haemagglutinin have been designated HA0 (Schwarz and Klenk, 1974).

HA is responsible for attachment of virus to receptors in the plasma membrane of the host cell. Cleavage of this protein into HA1 and HA2 has been found necessary for infectivity (Klenk et al., 1975). However, cleavage is not required for adsorption to host cells (Klenk et al., 1975; Lazarowitz and Choppin, 1975) implying that HA has some other, but as yet undetermined, function.

d) Neuraminidase (NA)

Like haemagglutinin, neuraminidase is also a glycoprotein, containing both type I and type II carbohydrate chains (Schwartz et al., 1977).

NA catalyses the removal of terminal N-acetyl neuraminic acid from specific glycoprotein substrates. It has been suggested that this enzyme mediates the release of virus from the host cell surface after multiplication (Gottschalk, 1965). However, monovalent antibodies which inhibit the enzymic activity were found not to interfere with virus release (Becht et al., 1971).

Influenza viruses do not contain neuraminic acid on their envelopes. This is thought to be due to digestion by viral NA (Klenk and Choppin, 1970; Klenk et al., 1970). In studies of ts influenza virus mutants with a defect in NA it

has been demonstrated that virions containing neuraminic acid residues were produced. These residues apparently served as receptors for the virus haemagglutinin, resulting in the production of large aggregates. This effect was overcome by the action of exogeneous bacterial neuraminidase. It was therefore proposed that the NA functions to produce particles which lack neuraminic acid, thereby avoiding aggregation, and that this function is essential for normal yields of progeny virus (Palese et al., 1974). Support for this mechanism has been obtained by growing virus in the presence of the NA inhibitor FANA (2-deoxy-2,3-dehydro-N1-trifluoracetylneuraminic acid). Growth of virus in the presence of this inhibitor caused extensive aggregation of progeny virions along with a 100-fold reduction in infectivity (Palese and Compans, 1976).

An additional function of NA has been reported by Schulman and Palese (1977). These authors found by recombination studies that NA of the influenza strain WSN was required for plaque production of influenza A viruses in MDBK cells. They postulated that removal of neuraminic acid may be a precondition for cleavage of HA and that by this mechanism NA may have another role in infectivity. However, this conclusion was not supported by the experiments of Bosch et al. (1979). These authors examined the infectivity of several influenza strains having different HAs but only the N1-NA (WSN possesses

N1-NA) or the Neq 1-NA. They found that cleavage of HA depended on its structure presumably in terms of its amino acid sequence, rather than on the NA type present.

In many animals influenza is an infection of the respiratory tract. This tract is lined with sialomucoproteins which could provide alternative sites of attachment for HA and so prevent the virus from reaching its target cells. It is known for example, that treatment of influenza with sialomucoproteins reduces infectivity (Shen and Ginsberg, 1968). Therefore, a possible function for NA in the infection of animals could be to free the virus from these proteins.

e) Matrix Protein

Matrix protein is the most abundant protein in the virion (Compans et al., 1970; Schulze, 1970; Skehel and Schild, 1971). As already described, M protein is an important structural feature of the virion, forming a shell of protein enclosing the ribonucleoprotein complexes. Growth of influenza virus at 39°C results in the production of progeny virions with a reduced M protein content (Kendal et al., 1977). Such particles were found to be fragile, implying that M protein has a role in the stabilization of virions.

Laver (1973) and Schulze (1973) have envisaged M protein as a filler, supporting the virus membrane and determining the shape. However, treatment with the detergent deoxycholate, which removes lipid and viral glycoprotein, results in the formation of cores, consisting of M protein surrounding the nucleocapsid (Skehel, 1971; Nermut, 1972; Schulze, 1972). This suggests that matrix protein is closely associated with the ribonculeoprotein complex (Reginster and Nermut, 1976).

Matrix protein may also play a role in virus assembly. This has been suggested by Choppin et al. (1972) and Compans and Choppin (1975). These authors proposed that M protein provided a recognition site, allowing the nucleocapsid to align with specific areas of the cell membrane from which the progeny virus will later "bud" (see below). At the same time the M protein would deny host cell protein access to these areas of membrane.

A further function of the M protein is linked to the sensitivity of some strains to amantadine (Lubeck et al., 1978; Hay et al., 1979). The mechanism of action of amantadine is unknown., but it is thought to inhibit some early event in the virus replication cycle (Kato and Eggers, 1969; Skehel et al., 1977). Thus M protein prior to viral synthesis is either involved in this event and is the

molecule on which amantadine acts or else the M protein of resistant strains is able to act as a barrier preventing the drug reaching its target.

f) Non-Structural Proteins

NS1 becomes associated with the nucleolus of infected cells (Dimmock, 1969; Krug and Soeiro, 1975). It has been observed that influenza infection results in inhibition of the processing of ribosomal RNA (rRNA) precursors; although the synthesis of 45S rRNA continues (Stephenson and Dimmock, 1974; Krug and Soeiro, 1975). This has prompted suggestions that NS1 is involved in this inhibition. However, in WSN infected MDBK cells there is five times less nucleolar NS1 than in similarly infected HeLa cells and yet the inhibition of rRNA processing is more pronounced (Krug and Soeiro, 1975).

NS1 is also found associated with polysomes in the cytoplasm (Pons, 1972; Compans, 1973) though this association is thought to be artefactual (Krug and Etkind), 1973).

A further possible function for NS1 has been found by Almond et al. (1978). These authors isolated a mutant of fowl plague virus with a temperature sensitive lesion in the NS1 gene. At the non-permissive temperature levels of

vRNA synthesis were greatly reduced. This implies that NS1 has a role in vRNA synthesis. Some support for this conclusion is provided by Maeno et al. (1979). These authors have produced evidence that the production of NS1 and its appearance in the cytoplasm can be correlated with an increase in vRNA synthesis. However, the data of Almond et al. (1978) may be open to a different interpretation in the light of the information that vRNA segment 8 codes not only for NS1 but also for NS2 (Inglis et al., 1979).

To increase uncertainty about the possible function of NS1 it has been found that the paracrystalline cytoplasmic inclusions which appear late in the infected cell are composed almost entirely of this protein (Morrongiello and Dales, 1977; Shaw and Compans, 1978; Nakamura et al., 1978). The ability of NS1 to undergo self-crystallisation plus the fact that it can be obtained in large amounts (1 mg per 10^9 BHK21-F cells) should facilitate both crystallographic analysis and studies into possible enzymatic and/or regulatory functions.

The smaller non-structural protein (NS2) appears late in infection, is predominantly cytoplasmic (after 3 h p.i.) (Lamb et al., 1978), and as yet has not been ascribed any functional significance.

5. Multiplication of influenza viruses

a) Attachment to the host cell

The process of infection of susceptible cells is initiated by attachment of virus particles via the haemagglutinin polypeptide to receptors on the cell surface (Dales and Choppin, 1962; Choppin and Compans, 1975). These cell surface receptors possess a terminal neuraminic acid and are most likely sialoglycoproteins (Suttijit and Winzler, 1971; Steck, 1974).

b) Penetration of the virus into the cell

Attachment of the virus to the cell surface receptor is presumably the trigger for penetration into the cell. However, exactly how such penetration is achieved is not certain. A possible mechanism is by fusion of the viral and plasma membranes. Evidence that lipid containing viruses can fuse with cells comes mainly from studies with paramyxoviruses (Apostolov and Almeida, 1972; Morgan and Howe, 1968; Zhdanov et al., 1963; Scheid and Choppin, 1974). However, the only evidence suggesting that influenza virus fuses with its host cell comes from the electron microscopic studies of Morgan and Rose (1968) and this evidence has been challenged by several other studies using

the electron microscope (Dales and Choppin, 1962; Dourmashkin and Tyrrell, 1970, 1974; Skehel et al., 1977; Patterson et al., 1979). Although these latter studies do not rule out fusion events they indicate that the majority of influenza particles enter cells by viropexis, an endocytotic event in which the whole virus is engulfed in a pinocytotic vesicle. Such a mechanism was first proposed by Fazekas de St. Groth (1948).

Pinocytosis can be divided functionally into macro- and micropinocytosis (Allison and Davies, 1974). Macropinocytosis is energy dependent and inhibited at low temperatures, whereas micropinocytosis is energy independent and occurs at 4°C (Allison and Davies, 1974). Cellular entry of influenza viruses at 4°C has been observed by electron microscopy (Hackemann et al., 1974) and in experiments employing radiolabelled virus (Stephenson and Dimmock, 1975; Stephenson et al., 1978). Furthermore, entry of influenza particles into cells still occurs under conditions where macropinocytosis is inhibited (Patterson et al., 1979). These data indicate that energy independent micropinocytosis is the probable mechanism for entry of influenza virus into cells.

Of much greater uncertainty than how the virus enters the cell is how the virus is uncoated and how the genome is prepared for transcription. Fazekas de St. Groth (1948)

originally proposed that the virus containing endocytotic vacuoles fused with cell lysozymes, resulting in partial digestion of the vacuolar and viral membranes and liberation of the viral core. However, this seems unlikely in view of the probable effects lyposomal enzymes would have on viral RNPs. An alternative is that pinocytotic vesicles fuse with internal membranes of the cell and release the virus, but no firm evidence exists to support this suggestion. However, it is known that some of the infecting influenza virion components become nucleus associated (Hoyle and Frisch-Niggemeyer, 1955; Hoyle and Finter, 1955; Stephenson and Dimmock, 1975). Using the technique of 4°C infections, Hudson et al. (1978) have shown that most of the input influenza virions which are taken up by cells rapidly come into contact with the nucleus. A ribonucleo-protein complex, comprising the virion RNA and much of the virion protein, then enters the nucleus, apparently leaving the phospholipids in the cytoplasm.

There is little evidence to suggest how the genome of influenza is made ready for the first stages in multiplication. However, it has been observed that nuclear associated viral RNPs have a tendency to shift to a lower density, implying a gradual stripping of the RNA core with time. This suggests that prior to transcription of the virion RNA, a considerable amount of non-essential protein may be removed (Hudson et al., 1978).

c) Time course of appearance of viral polypeptides

Influenza viruses replicate sub-optimally in most cultured cell lines (Henle et al., 1955). Such low yielding or abortive infections provide poor experimental systems for studying the processes of viral replication. However, the influenza strain fowl plague virus grows efficiently in primary chick embryo fibroblast cells (CEF). The release of progeny virus begins between 3 and 4 hrs after infection and in a one-step growth curve release of progeny virus is complete by 8 hrs (Borland and Mahy, 1968). Therefore, much of the following discussion is based on studies from this system.

The genome of influenza is of negative polarity and therefore must be transcribed into functional mRNA before replication can commence. This mRNA must be subject to some form of control as witnessed by viral polypeptide synthesis.

Pulse labelling of CEF cells at different times after infection with radioactive amino acids has shown influenza polypeptide synthesis to be regulated in two important aspects. First, different amounts of individual polypeptides are produced and second the virus polypeptides show differing time course of appearance.

Early in infection (1.5 to 2.5 h postinfection) NP and NS1

are synthesized in large amounts relative to M protein. However, from about 3 h p.i. the synthesis of M is amplified to become the most abundant viral polypeptide (Skehel, 1972, 1973; Inglis et al., 1976; Inglis and Mahy, 1979).

The three P proteins can also be detected early; although their synthesis remains at a low level throughout infection (Lamb and Choppin, 1976). Less clear is the time course of appearance of HA and NA. In FPV infected CEF cells HA has been detected as early as NSI but its rate of accumulation appears to increase later in infection (Skehel, 1972, 1973; Inglis et al., 1976; Bosch et al., 1978). NA is difficult to detect in pulse labelling experiments since it co-migrates with NP in many PAGE systems (Skehel, 1972; Minor and Dimmock, 1975; Lamb and Choppin, 1976; Inglis and Mahy, 1979).

This then provides a framework for discussion of influenza transcription and replication.

d) Composition of complementary RNAs (cRNA)

There are two types of virus specific complementary RNA, those which have poly(A) sequences at their 3' termini (Etkind and Krug, 1974) and those which lack added poly(A).

These populations are separated by affinity chromatography using oligo (dT)-cellulose and electrophoretic analysis has shown that both classes contain molecules complementary to all eight genomic RNA's (Hay et al., 1977). S1 nuclease treatment of hybrids formed between either polyadenylated cRNA or unpolyadenylated cRNA and virion RNA demonstrated the former class of cRNA to be smaller (see below) than the latter (Hay et al., 1977; Skehel and Hay, 1978). RNase T_1 oligonucleotide mapping and nucleotide sequence analysis revealed unpolyadenylated cRNAs to be complete transcripts of influenza genome whereas polyadenylated cRNAs lack at least 17 nucleotides from the 5' terminal region (Hay et al., 1977; Skehel and Hay, 1978).

Furthermore, the sequences of those regions of virion RNAs not represented in polyadenylated cRNAs are almost identical (Skehel and Hay, 1978) (see Fig. 1).

Incomplete transcripts could be produced by nucleolytic processing of complete transcripts or by terminating transcription prematurely. The latter alternative is more likely since in the absence of protein synthesis (see below) only incomplete transcripts are produced (Hay and Skehel, 1979). Suggestions have been made that the U_6 (Fig. 1) sequence represents a termination signal and that protein synthesis is required to modify the enzyme and allow transcription to proceed through this signal (Hay and Skehel, 1979).

Fig. 1

Nucleotide sequences of the conserved regions of influenza genome RNAs and the transcripts

Virus RNA

(3') HO-U-C-G-U-U-U-U-C-G-U-C-C ..... U-U-U-U-U-U-(--)*-G-G-A-A-C-A-A-A-G-A-U-G-Appp (5')

mRNA

(5') M^7G-A-G-C-A-A-A-A-G-C-A-G-G ..... ----$A_{(150)}$ -A-OH (3')

cRNA (non-poly A)

(5') A-G-C-A-A-A-A-G-C-A-G-G ..... A-A-A-A-A-A-(--)-C-C-U-U-G-U-U-U-C-U-A-C-U-OH (3')

From Hay and Skehel, 1979

*The sequences of nucleotides 14-16 at the 5' ends of virus RNA are variable

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e) Function of polyadenylated and unpolyadenylated cRNAs

Both types of cRNA function as efficient messenger RNAs (mRNA) in cell free protein synthesizing systems (Stephenson et al., 1977; Content, 1976). However, only polyadenylated cRNAs are associated with polysomes in vivo (Hay et al., 1977). Therefore, this class of cRNA probably functions as mRNA whereas unpolyadenylated cRNA (i.e. complete transcripts) functions as a template for virion RNA synthesis. Very little free cRNA is found in infected cells. It is either associated with polysomes or bound in the form of ribonucleoprotein complexes (Hay et al., 1977). The synthesis of cRNA occurs in two phases. The first, or primary transcription, lasts for about 30 min (Inglis and Mahy, 1979) after infection and involves transcription of the influenza genome by the virion-associated RNA polymerase (Bean and Simpson, 1973). The second, or secondary transcription is dependent on viral proteins synthesized as a result of primary transcription (see below). This stage is responsible for the majority of cRNA synthesis (Mark et al., 1979).

f) Primary transcription

The short time over which primary transcription occurs makes its study difficult. Bean and Simpson (1973) found that synthesis of cRNA was severely reduced by adding the drug cycloheximide (CM) (100 μg/ml) to cells at the time of infection. These authors concluded that the remaining cRNA synthesis was due to the activity of the virus-associated polymerase. In the presence of CM, synthesis of vRNA is suppressed (Scholtissek and Rott, 1970; Pons, 1973) and the amount of cRNA detected at 2.5 h post-infection is only 10-20% of the level found in untreated cells (Taylor et al., 1977). This low level remains unchanged for up to 5 h after infection (Taylor et al., 1977). Therefore CM, by inhibition of cellular protein synthesis, is thought to prevent secondary transcription of the influenza genome. However, CM does not completely suppress vRNA synthesis (Taylor et al., 1977; Mark et al., 1979). This implies that viral transcription in the presence of this drug is not confined to the inoculum vRNA but that there is some transcription of newly synthesized cRNA. The addition of actinomycin D (AMD) (2-10 μg/ml) along with CM eliminates this vRNA synthesis (Taylor et al., 1977; Mark et al., 1979). This combination of drugs also reduces levels of cRNA synthesis further than CM alone (Taylor et al., 1977; Mark et al., 1979). Thus, the use of CM and AMD or CM alone are the usual methods employed for the study of primary transcription.

In the presence of CM or CM plus AMD polyadenylated transcripts of all the influenza genes, except that coding for the NS2 protein (Inglis and Mahy, 1979), can be detected. Furthermore, these transcripts are present in roughly equimolar amounts (Hay *et al.*, 1977; Mark *et al.*, 1978, 1979). This is apparently not an artefact since a similar pattern of transcription is detected in normally infected cells during the first 30 min of infection (Hay *et al.*, 1977). Furthermore, translation either *in vitro* (Inglis and Mahy, 1979; Mark *et al.*, 1979) or by removal of the CM block (Lamb and Choppin, 1976; Inglis and Mahy, 1979) have shown these transcripts to be functional mRNAs.

When CM is added to cells at the time of infection cRNA synthesis is reduced to 5-15% of that in untreated cells. Of the residual cRNA synthesized 42% is found in the nucleus. If AMD is used in addition to CM, cRNA synthesis is again reduced to about 15% of that in untreated cells, but of the residual cRNA synthesized 79% is found in the nucleus (Mark *et al.*, 1979). This evidence suggests that primary transcription occurs in the nucleus. However, in the studies of Mark *et al.* (1979) radioactive vRNA or radioactive complementary DNA (cDNA) were used as probes for detecting cRNA (cDNA was prepared from purified viral cRNA by the use of reverse transcriptase). Although sensitive,

such methods of detection measure only accumulation of cRNA rather than its rate of synthesis at a particular time. Thus it is possible that primary transcription occurs in the cytoplasm and that the high proportion of cRNA found in the nucleus under conditions of AMD and/or CM inhibition is the result of transport.

As already mentioned the use of CM and AMD have shown a characteristic of primary transcription to be that transcripts of all the influenza genes are produced in roughly equal amounts. These, when added to an *in vitro* translation system, direct the synthesis of all viral polypeptides in roughly equal amounts (Inglis and Mahy, 1979; Mark *et al.*, 1979). These data imply that there is no transcriptional control of primary transcription.

g) Secondary Transcription

Secondary transcription comprises the synthesis of complete unpolyadenylated transcripts as well as further synthesis of polyadenylated cRNA s. Synthesis of these two classes of cRNA appears to be separately controlled. Firstly after the first hour of infection, synthesis of polyadenylated cRNA always exceeds that of unpolyadenylated molecules (Hay *et al.*, 1977). Secondly, synthesis of unpolyadenylated cRNA reaches a maximum 1 to 5 h post-infection, whereas

polyadenylated cRNA is produced maximally at about 2 h after infection. Thirdly, the production of unpolyadenylated cRNA is more drastically curtailed relative to poly A-containing cRNA later in infection.

i) Secondary Transcription: Polyadenylated cRNA Synthesis

Equal transcription of the genomic RNA segments changes dramatically 30 min after infection. Between 30 and 90 min transcripts 5 and 8 are synthesized preferentially. This changes again such that by 2.5 h post-infection transcripts 4, 5 and 7 are made in greatest amounts. In contrast transcripts 1 to 3 are synthesized in relatively small amounts throughout infection (Hay et al., 1977). In addition, the maximum rate at which the various polyadenylated cRNAs are produced occurs at different times during infection (Hay et al., 1977; Barrett et al., 1978, 1979).

1. The synthesis of transcript 8 increases sharply after 1 h and reaches a maximum around 1.5 h, thereafter it decreases rapidly to about 10% of maximum by 3.5 h.

2. The synthesis of transcripts 1, 2, 3 and 5 is 50% of maximum around 1.25 h. It reaches a peak at 2 h and is still about 20% of maximum at 4.5 h.

3. The peak in the synthesis of transcripts 4, 6, 7 and 10 is between 2 and 2.5 h. By 4.5 h this synthesis is approximately 30% of maximum.

4. Synthesis of transcript 9 increases throughout infection.

The synthesis of the various polyadenylated cRNA's correlates well with the appearance and relative abundance of the polypeptides for which they code. NP and NS1 are the most abundant polypeptides early in infection. These are coded for by transcripts 5 and 8 respectively. As previously described these transcripts are synthesized preferentially between 30 and 90 min post-infection. Such correlations point to the controlled synthesis of polyadenylated cRNA as being responsible for determining the amounts of viral polypeptides synthesized during infection. Additional support for this theory derives from experiments in which viral mRNA's were extracted at different times during infection and translated *in vitro* (Inglis & Mahy, 1979).

A mechanism which could explain such control is selective initation of transcription. However, there is evidence against this possibility. Firstly, primary transcription results in equal production of polyadenylated cRNA's (Hay *et al*., 1977, 1978; Inglis and Mahy, 1979; Mark *et al*., 1979). Secondly, sequence determination has shown that the

first twelve nucleotides at the 3' termini of all the genome RNAs are the same and it is possible that this conserved sequence may represent the transcriptase binding site (Hay and Skehel, 1979). Should this prove to be the case then no structural basis for selective initiation is presented in this region. Similarly, further comparisons of the sequence of the first 30 nucleotides of virion RNAs 4 to 8 do not indicate any obvious features that would correlate with for example, the preferential transcription of RNAs 5 and 8 early in secondary transcription (Hay and Skehel, 1979).

An alternative mechanism is that the composition of enzymes involved in primary and secondary transcription are different (Hay et al., 1977; Hay and Skehel, 1979; Inglis and Mahy, 1979). Evidence in favour of this hypothesis is that protein synthesis is required for secondary transcription. Furthermore, a ts mutant of FP/Weybridge has been observed which is defective in secondary transcription at the non-permissive temperature but not in primary transcription (Ghendon et al., 1975). Should such a mechanism operate then the composition of the transcriptase would have to alter at least twice, once for the preferential transcription of segments 5 and 8 and again for the amplication in the synthesis of transcripts 4 and 7. A suggestion that this might happen is seen by the addition of cycloheximide at 1.5 h post-infection, when the

amplification in the synthesis of M protein is prevented (Inglis and Mahy, 1979). How the composition of the transcriptase might be altered during infection and exactly how such alteration results in preferential transcription of the viral genome are at present unknown.

Alterations in the composition of transcriptase enzymes may not however, represent the complete answer to the problem of transcriptional control. The host cell does have an influence on the amounts of some polyadenylated cRNA species (Bosch et al., 1978) and on the time of appearance of viral protein (Minor et al., 1979). These host cell effects will be discussed later.

ii) Secondary Transcription: Unpolyadenylated cRNA Synthesis

In contrast with the disproportionate synthesis of the different polyadenylated cRNA molecules, unpolyadenylated cRNA's, with the exception of transcripts 9 and 10, are present in similar amounts until 3 h post-infection (Hay et al., 1977). Furthermore, the relative rates at which unpolyadenylated cRNA's are synthesized is constant until 2 h post-infection. However, at later times the pattern of synthesis tends to reflect that of polyadenylated cRNAs. In particular, the synthesis of transcript 8 is markedly curtailed (Hay et al., 1977). A possible mechanism for unpolyadenylated cRNA production is

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that proteins synthesized during infection modify the transcriptase allowing it to proceed beyond the normal (premature) termination signal (Hay and Skehel, 1979).

iii) Secondary Transcription - Virion RNA synthesis

Estimates of the time course of progeny virion RNA (vRNA) synthesis have differed. Scholtissek and Rott (1970) found vRNA synthesis to be maximal 3 h post-infection. Hay et al. (1977) found vRNA synthesis to be at its peak between 1.5 and 2 h post-infection. It then decreased steadily so that by 5 h it was about 6% of maximum. It is difficult to reconcile the results obtained by these two groups of workers, since they both used pulse-labelling techniques in the same virus-cell system.

A different approach to the problem of estimating the time course of vRNA synthesis has been to measure the kinetics of hybridization of unlabelled RNA from infected cells to specific radioactive probes (Taylor et al., 1977; Barrett et al., 1978, 1979; Mark et al., 1978). The probe used was complementary DNA (cDNA) synthesized from a vRNA template by reverse transcriptase. Although this hybridization technique is extremely sensitive, it can only measure total accumulated RNA and not the rate of synthesis at any particular time.

Using this approach, closely similar results have been obtained for the kinetics of vRNA accumulation in MDCK cells infected with influenza strain WSN (Taylor et al., 1977; Mark et al., 1978) and in CEF cells infected with fowl plague virus (Barrett et al., 1978, 1979). The first significant increase in the amounts of vRNA occurs between 1 and 2 h post-infection, after which there is a steady increase in the amount of vRNA up to 6 h. Early in infection the amount of cRNA is greater than vRNA. However, at 2.75 h the level of vRNA increases relative to cRNA, such that it becomes the majority species (Taylor et al., 1977).

New protein synthesis is required for the appearance of vRNA in infected cells (Scholtissek and Rott, 1970; Taylor et al., 1977). However, the exact details of this requirement are not known.

h) Location of virus specific RNAs and their sites of synthesis

i) Site of cRNA synthesis

There is circumstantial evidence that cRNA synthesis occurs in the cytoplasm, in that the majority of cRNA is associated with the cytoplasm at all times during infection (Avery, 1974; Hay et al., 1977; Taylor et al., 1977) and newly synthesized cRNA is mainly associated with cytoplasmic fractions after

pulse-labelling with ^{3}H-uridine (Hay et al., 1977). In addition the majority of virus specific RNA-dependent RNA polymerase activity, which is capable of synthesizing cRNA in vitro, is found in the microsomal fraction of infected cells (Ho and Walters, 1966; Scholtissek and Rott, 1969; Skehel and Burke, 1969; Ruck et al., 1969; Mahy, 1970). However, considering the time course of appearance of this polymerase activity it is probable that much of it is destined for inclusion into progeny virions rather than for synthesizing cRNA.

In contrast to evidence that cRNA synthesis is cytoplasmic, it has been found that the polyadenylated form of cRNA contains internal N^{6}-methyladenosine residues (Krug et al., 1976). Such modified bases have been detected in mRNAs synthesized in the nucleus but not in mRNAs of viruses that are synthesized in the cytoplasm (Abraham et al., 1975; Desrosiers et al., 1975; Furuichi et al., 1975; Lavi and Shatkin, 1975; Shatkin and Both, 1976). Some support for the nucleus being the site of polyadenylated cRNA synthesis has come from Barrett et al. (1979). Using low concentrations of AMD (0.1 µg/ml) which suppressed but did not abolish secondary transcription, these authors found that polyadenylated cRNAs accumulated only in the nucleus during the period 2 to 4 h p.i. In untreated cells at these times amounts of polyadenylated cRNAs increased in the cytoplasm but decreased in the nucleus.

Although this data is consistent with the nucleus being the site of polyadenylated cRNA synthesis, and that AMD acts to prevent its transport to the cytoplasm, there are a number of problems. Firstly, by using radiolabelled vRNA as a probe for cRNA, Barrett *et al*. (1979) could measure only accumulation of RNA and not synthesis. Therefore the results are open to the interpretation that polyadenylated cRNAs are synthesized elsewhere and then transported to the nucleus. Secondly, the use of any inhibitor is unfortunately open to the critism that it may generate an artefactual situation. That treatment of cells with 0.1 μg/ml of AMD reduces total amounts of polyadenylated cRNA by over 90% (at 4.5 h p.i.) compared with untreated cells (Barrett *et al*., 1979) serves to emphasize this point.

ii) Site of vRNA synthesis

Virus specific RNA dependent RNA polymerases isolated from the microsomal fraction of infected cells synthesize only cRNA *in vitro* (Mahy and Bromley, 1970; Hastie and Mahy, 1973). However, Mahy *et al*. (1975) isolated a virus specific RNA polymerase from the nuclear fraction which synthesized small amounts of vRNA *in vitro*. Furthermore, Assadullaef *et al*. (1975) have found an RNA synthesizing complex with replicase properties in the nucleoplasm of fowl plague infected Ehrlich ascites tumour cells. Although extraction of polymerases

may lead to altered specificity through loss of labile or weakly bound components, these results imply the nucleus to be the site of vRNA synthesis. This conclusion is in agreement with data produced by Avery (1974) who after pulse labelling cells with ^{3}H-uridine found high levels of vRNA in the nucleus relative to those in the cytoplasm. However, the comparatively long labelling times used by this author (1.5 h) means the results are also open to the interpretation that vRNA synthesis is cytoplasmic and that the high levels in the nucleus are due to transport and accumulation.

6. Influence of the host cell in influenza virus multiplication

a) The nuclear step?

Influenza virus has a special requirement for the host cell nucleus. The nucleus appears to be the target for the RNA of the infecting virus (Stephenson and Dimmock, 1975; Hudson et al., 1978) and during infection virus-specific products are found there (Breitenfeld and Schafer, 1957; Fraser, 1967; Dimmock, 1969; Taylor et al., 1969, 1970; Krug and Etkind, 1973; Lazarowitz et al., 1971; Avery, 1974; Krug and Soerio, 1975; Hay et al., 1977; Taylor et al., 1977). Influenza virus, unlike other RNA viruses (not including viruses having a DNA intermediate in their replication cycle) will not grow in enucleate cells (Follet et al., 1974; Kelly et al., 1974) or in cells which have been treated with inhibitors of DNA function (Barry et al., 1962; Barry, 1964; Rott et al., 1965; Nayak and Rasmussen, 1966). One of these inhibitors of DNA function is the drug α-amanitin. This inhibits influenza replication but only when it is added at the beginning of infection (Rott and Scholtissek, 1970; Mahy et al., 1972). Viral transcription or vRNA replication is not detected when α-amanitin (20 μg/ml) is added at the time of virus adsorption. However, these functions are completely insensitive to the drug if it is added 1.5 h after infection (Mark et al., 1979).

α-Amanitin inhibits DNA dependent RNA polymerase II, both *in vivo* and *in vitro* (Chesterton and Butterworth, 1971; Fuime and Wieland, 1970; Hastie and Mahy, 1973; Kedinger *et al.*, 1970; Novello and Stirpe, 1970). This enzyme synthesises mRNA (Roeder and Rutter, 1969, 1970). Therefore, it has been proposed that host cell polymerase II is required for initiation of viral transcription (Mahy *et al.*, 1972; Plotch and Krug, 1977; Lamb and Choppin, 1977; Spooner and Barry, 1977). Evidence supporting this suggestion has been obtained using a mutant cell line with an α-amanitin resistant RNA pol II. Influenza virus polypeptide synthesis occurs in this cell line in the presence of α-amanitin (Lamb and Choppin, 1977; Spooner and Barry, 1977). There are at least three mechanisms to explain the involvement of RNA polymerase II in initiation of viral transcription. The first is that RNA polymerase II transcribes the influenza genome after infection. This is unlikely since the virus possesses an RNA polymerase. Secondly, an α-amanitin sensitive subunit of RNA polymerase II might be required by the viral polymerase and thirdly, a product of RNA polymerase II may be required to initiate viral transcription.

Transcription of the influenza genome *in vitro* has given some possible clues about the relationship of RNA polymerase II with viral RNA synthesis. In the presence of Mg^{2+} viral RNA can be transcribed into polyadenylic acid containing cRNA.

However, with the WSN and fowl plague strain of influenza the yields of cRNA are low unless the dinucleotides ApG or GpG are added to the system (McGeoch and Kitron, 1975; Content *et al.*, 1977; Plotch and Krug, 1977, 1978). The 5' sequence of cRNA is ApGpC (Hay *et al.*, 1978, Hay and Skehel, 1979) which is complementary to the GpCp UoH 3' terminus of vRNA (Hay and Skehel, 1979). The use of radio-labelled ApG has shown that it is bound at the 5' terminus of cRNA synthesized *in vitro* (Plotch and Krug, 1978). Therefore ApG might initiate transcription *in vitro* by binding to the 3' terminus of vRNA. Initiation by GpG can also be explained by this model. GpG could hydrogen bond to the penultimate GpC sequence at the 3' end of vRNA When a GpG dinucleotide is bound to a polynucleotide G-to-G interactions between the two are as strong as Watson-Crick G to C interactions (Lewis *et al.*, 1975).

Transcription of WSN viral RNA *in vitro* is also stimulated by globin mRNA and several other naturally occurring eukaryotic messengers (Bouloy *et al.*, 1978). cRNA synthesized *in vitro* in the presence of globin mRNA possess a 5' methylated cap structure even though such structures are not synthesized *de novo* in the *in vitro* system (Bouloy *et al.*, 1978). This suggests the cap and possibly other 5' terminal sequences of globin mRNA are spliced onto the 5' end of viral cRNA during transcription.

The ability of an RNA to prime viral transcription is dependent on the possession of a 5' methylated cap structure. Ribosomal RNA (rRNA) or transfer RNA (tRNA) will not act as efficient primers and neither will globin mRNA if the cap is removed (Bouloy et al., 1978). On the basis of these data it is proposed that host cell polymerase II synthesizes a mRNA which initiates viral transcription. Mark et al. (1979) use this hypothesis to explain the early sensitivity and the late (1.5 h) refractoriness of cRNA synthesis to α-amanitin. These authors suggest at the time of infection the amount of primer is insufficient for viral RNA synthesis so that de novo synthesis of primer mRNA is required. At later times during infection enough primer cell mRNA molecules have accumulated to make further synthesis unnecessary. An implication of this hypothesis is that infection stimulates the synthesis and/or accumulation of primer molecules. In this respect it is interesting to note that an increase in host RNA synthesis has been observed in CEF cells shortly after infection with fowl plague virus (Borland and Mahy, 1968; Mahy, 1970). However, experiments to investigate the nature of the RNA synthesis have not been carried out.

A variation of the above hypothesis arises from the suggestion that the composition of viral polymerase alters during infection. Thus only the viral polymerase active early in infection (i.e. the virion associated polymerase) requires

primer mRNAs. This requirement then ceases with changes in viral polymerase composition.

A model for the mechanism of action of the putative primer mRNAs has not been fully described. However, it is possible to envisage 5' terminal regions of primer mRNAs being complementary and hybridizing to the 3' terminus of vRNA. Following such hybridization the remaining single stranded region of the mRNA would be cleaved leaving a double stranded region on the vRNA from which transcription could be initiated.

b) Additional Host-Cell/Virus interactions?

The requirement of RNA polymerase II in influenza replication is only the first of several ways in which the host cell can influence the expression of the viral genome. Evidence for this statement comes from the results of treating infected cells with host specific drugs and from the study of non-permissive infections.

Actinomycin D (AMD) inhibits all detectable viral polypeptide synthesis (Skehel, 1973) but at lower critical concentrations of the drug only the synthesis of HA, NA and M are inhibited (Minor and Dimmock, 1975, 1977). AMD is only effective when added early (i.e. before 1.5 h post-infection) in infection

(Barry, 1964; Gregoriades, 1970) and its target seems to be within the host cell in so much as it does not effect virion associated polymerase activity *in vitro* (Chow and Simpson, 1971). Treatment of infected cells with camptothecin has a similar effect to that of a critical concentration of AMD in that only the synthesis of HA, NA and M are inhibited (Minor and Dimmock, 1975, 1977). How do critical concentrations of AMD or camptothecin preferentially inhibit these proteins? AMD has been reported to inhibit ribosomal RNA synthesis at slightly lower concentrations than mRNA synthesis (Perry, 1969; Craig, 1971). Camptothecin inhibits ribosomal RNA (rRNA) synthesis but leaves 10% of mRNA synthesis intact (Wu *et al.*, 1971; Abelson and Penman, 1972). From these observations it was proposed that influenza replication required some aspect of mRNA synthesis for the expression of the P proteins, NP and NS, and in addition, some aspect of rRNA synthesis for HA, NA and M protein synthesis (Minor and Dimmock, 1975, 1977). Although this is an attractive possibility it must be pointed out that there are other suggestions to explain the effect of AMD on influenza replication. Mark *et al.* (1979) observed that of the small amount of polyadenylated cRNA synthesized in the presence of AMD at 2 μg/ml, over 90% was confined to the nucleus. Barrett *et al.* (1979) found that in cells treated with 0.1 μg/ml of AMD (the same as the critical concentration reported by Minor and Dimmock, 1975, 1977), polyadenylated

cRNAs coding for HA and M accumulated in the nucleus but decreased in the cytoplasm. Whereas over the same period amounts of the polyadenylated cRNAs coding for NP and NS1 increased in both the nucleus and the cytoplasm. Therefore, it has been proposed that AMD inhibits influenza replication by confining newly synthesized polyadenylated viral transcripts to the nucleus. High concentrations (> 0.1 μg/ml) of the drug are seen as inhibiting the transport of all species of polyadenylated cRNA whereas low, critical concentrations inhibit the transport of only certain species (Mark et al., 1979; Barrett et al., 1979).

Besides inhibition by camptothecin and low, critical concentrations of AMD the synthesis of HA, NA and M is also inhibited if cells are irradiated with ultra-violet light (UV) prior to infection (Mahy et al., 1977; Minor and Dimmock, 1977). UV light appears to block amplification in the synthesis of the polyadenylated transcripts coding for HA, NA and M (Mahy et al., 1977). This block is due to specific damage of the host cell and can be reversed if the cells are allowed to photoreactivate by exposure to visible light subsequent to UV irradiation (Mahy et al., 1977). The fact that this recovery process is inhibited by caffeine (Mahy et al., 1977) a compound known to block DNA repair (Cleaver and Thomas, 1969; Doman and Rauth, 1969; Fiyiwara and Kondo, 1972; Kihlman et al., 1974) suggests

that amplification in the synthesis of HA, NA and M may in some way be dependent on functional host cell DNA.

c) Non-permissive infections

Studies of non-permissive infections have revealed additional ways in which the host cell can influence influenza replication. Non-permissive infections occur (a) where the number of infectious particles produced relative to the number of particles causing haemagglutination are reduced, and (b) where the infectivity : HA ratio is normal but the total yield of virus is reduced. Examples of the former group are PR/8 and swine virus strains in CEF cells (Klenk et al., 1975), WSN in MDBK cells (Lazarowitz and Choppin, 1975), FPV in L-cells (Franklin and Breitenfeld, 1959; Rott and Scholtissek, 1963; Ghandhi et al., 1971), and FPV in Ehrlich ascites tumour cells (Bukrinskaya et al.,1978). An example of the second group is found in WSN infection of certain HeLa cells (Caliguiri and Holmes, 1979).

There are two more minor groups of non-permissive infection. One (c) where the yields of HA and the ratio of infectivity to HA are both lower than those found in permissive infections; an example being NWS infection of L-cells (Minor et al., 1979) and the second (d) where there are no detectable progeny virions produced. An example of this is FPV infection of KB cells (Valcavi et al., 1978).

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There are two more minor groups of non-permissive infection. One (c) where the yields of HA and the ratio of infectivity to HA are both lower than those found in permissive infections; an example being NWS infection of L-cells (Minor et al., 1979) and the second (d) where there are no detectable progeny virions produced. An example of this is FPV infection of KB cells (Valcavi et al., 1978).

This classification does not denote any common cause of non-permissiveness within a particular group. Indeed examination of the causes reveals a different point at which influenza replication is blocked for almost every non-permissive virus/cell system. Thus in group (a), PR8 and swine infection of CEF cells and WSN infection of MDBK cells are non-permissive because newly synthesized HA remains uncleaved (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Progeny virus particles with uncleaved HA can still absorb to cells but are not able to replicate (Klenk et al., 1975; Lazarowitz and Choppin, 1975). The fowl plague virus (FPV)/Ehrlich ascite tumour cell system of group (a) is non-permissive because the progeny virus produced is unstable owing to a reduction in the amount of M protein (Bukrinskaya et al., 1978). In FPV infections of L-cells there is an accumulation of viral NP in the nucleus (Franklin and Breitenfeld, 1959; Rott and Scholtissek, 1963). However, this is probably a reflection of some other event rather than the primary cause of the infection being non-permissive. The identity of this primary cause is not certain. Avery (1975) found vRNA synthesis to be blocked whereas Bosch et al. (1978) found normal levels of vRNA but a reduction in polyadenylated transcripts 6 and 7. In the examples given for other groups of non-permissive infection different mechanisms can be seen in operation. Thus in the WSN/HeLa cell system (Group b) the final stages of virus

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particle maturation at the plasma membrane are blocked (Caliguiri and Holmes, 1979) while in FPV infection of KB cells (Group d) no M protein is synthesized (Valcavi et al., 1978). These examples all serve to indicate that there are numerous host-influenced steps affecting influenza at almost every point in its replication cycle and that negotiation of each of these steps requires a finely "tuned" interaction between the host cell and the virus. This conclusion is similar to that reached by Minor et al. (1979) from work showing that different virus strains exhibit varying growth characteristics in the same host cell type.

7. Sites of synthesis of viral proteins

Cell fractionation studies and analysis of viral proteins and mRNAs suggest the site of synthesis of the glycoproteins (HA and NA) and M protein is the rough endoplasmic reticulum (Compans, 1973; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974; McGeoch et al., 1976).

The synthesis of the P proteins, NP and NS1 does not appear to be associated with cytoplasmic membranes since the RNAs coding for these proteins are found mainly with free ribosomes (Hay, 1974; Hay et al., 1977).

8. Movement of viral proteins between compartments of the host cell

Movement of viral proteins between compartments of the host cell has been studied using the techniques of cell fractionation and indirect immunofluorescence. Cytoplasmic fractionation procedures follow the method of Caligiuri and Tamm (1970). This involves centrifugation of disrupted cells through discontinuous sucrose gradients to produce discrete bands of membraneous material. By this technique it is possible to distinguish rough and smooth endoplasmic reticulum, a plasma membrane fraction, a fraction containing free ribosomes and a soluble fraction. The fractions can be identified by electron microscopy (Compans, 1973; Klenk et al., 1974). and by their enzyme content (Hay, 1974). However, this technique is subject to problems of cross-contamination of fractions, both by viral protein reassociating with adjacent fractions and by membranes resedimenting at different densities (Compans, 1973).

In view of its essential nature in influenza replication, movement of viral protein into one particular compartment of the cell, the nucleus, is of special interest. Movement of viral protein into this organelle has been studied using immunofluorescence (Breitenfeld and Schafer, 1957; Dimmock, 1969; Maeno and Kilbourne, 1970; Oxford and Schild, 1975)

and nuclear/cytoplasmic fractionation techniques such as Dounce homogenization (Krug and Etkind, 1973; Hay, 1974; Hay and Skehel, 1975) or the detergent disruption technique of Tsai and Green (1973) (Hudson and Dimmock, 1977; Flawith and Dimmock, 1979).

a) Haemagglutinin and neuraminidase

Cell fractionation studies suggest that after synthesis on the rough endoplasmic reticulum HA and NA migrate to the plasma membrane via the smooth internal membranes (Compans, 1973; Stanley et al., 1973; Hay, 1974; Klenk et al., 1974). In the course of migration these polypeptides undergo post-translational modifications. These involve sequential glycosylation at the rough endoplasmic reticulum and at the smooth internal membranes (Compans, 1973; Stanley et al., 1973) and for HA, proteolytic cleavage which occurs at the smooth internal membranes (Klenk et al., 1974) or at the plasma membrane (Lazarowitz et al., 1971; Hay, 1974).

Immunofluorescence studies suggest that HA and NA remain in the cytoplasm throughout the course of infection (Breitenfeld and Schafer, 1957; Dimmock, 1969; Maeno and Kilbourne, 1970; Oxford and Schild, 1975).

b) Matrix Protein

Within two minutes of its synthesis matrix (M) protein is found associated with all cell membrane fractions (Klenk et al., 1974; Meier-Ewert and Compans, 1974; Hay and Skehel, 1975). However, whether M protein migrates to the nucleus is debatable. M protein has been reported to be entirely cytoplasmic in MDCK cells infected with either FP/R (Mahy et al., 1980) or WSN (Krug and Etkind, 1973) and in HeLa cells infected with WSN (Krug and Soeiro, 1975). On the other hand, M has been observed to accumulate in the nuclei of WSN or FP/R infected CEF cells (Gregoriades, 1973; Hay and Skehel, 1975; Flawith and Dimmock, 1979), while in another system (strain X31 in MDBK cells) M antigen is present in the nuclei of some but not all cells (Oxford and Schild, 1975).

c) Non-structural protein 1 (NS1)

After synthesis some NS1 migrates to the nucleus (Taylor et al., 1969, 1970; Lazorowitz et al., 1971; Hay and Skehel, 1975) and accumulates in the nucleolus (Dimmock, 1969; Krug and Soeiro, 1975). NS1 also migrates back out of the nucleus, but at a rate 2 to 3 fold less than observed with M and NP (Flawith and Dimmock, 1979).

A curious property of NS1 is that late in infection, crystalline aggregates of the protein are found in the

cytoplasm of infected cells (Morrongiello and Dales, 1977; Nakamura et al., 1978; Shaw and Compans, 1978). The function of such aggregates or indeed of the NS1 monomers is unknown.

d) Nucleoprotein (NP)

Studies using direct fractionation of nuclei and cytoplasm have shown that after synthesis in the cytoplasm, NP migrates rapidly to the nucleus, and in particular to the nucleoplasm (Krug and Etkind, 1973; Hay and Skehel, 1975). Most of this nuclear NP is in the form of RNPs (Krug and Etkind, 1973). Kinetic studies have shown there to be a 45 min time lag between NP synthesis and its incorporation into virions (Hay and Skehel, 1975). Therefore, it is conceivable that before incorporation, NP passes through the nucleus. In this respect it has been demonstrated that a proportion of nuclear-associated NP does migrate back to the cytoplasm (Flawith and Dimmock, 1979). However, there is no hard evidence to show that nuclear NP contributes to virions.

e) P Proteins

There is uncertainty about the intracellular location of P proteins. P Proteins have been found in the nuclei of WSN infected MDCK cells (Krug and Etkind, 1973) but only in

trace amounts in the nuclei of FPV infected chick embryo cells (Hay and Skehel, 1975). One problem in studying the intracellular location of P proteins is that they are synthesized in low amounts relative to other viral proteins. Another problem is that the polyacrylamide gel system used by both Hay and Skehel (1975) and Krug and Etkind (1973) could not resolve P proteins into three components. The former could resolve two bands while the latter could only resolve one. This makes interpretation of the results difficult since the three P proteins may behave differently within the infected cell.

9. Assembly and release of virus particles from the cell

Early electron microscopic studies indicated that influenza virus particles were not formed inside the cell but at the cell surface (Murphy and Bang, 1952). Later studies using cell fractionation techniques as well as the electron microscope have provided suggestive evidence for the order in which viral components associate at the cell surface prior to maturation. Apparently, the first event is incorporation of HA and NA into an area of the host cell plasma membrane, (Compans and Dimmock, 1969; Bachi *et al.*, 1969; Compans *et al.*, 1970; Hay, 1974) followed by attachment of M protein to the plasma membrane bearing the viral glycoproteins (Hay, 1974). It is difficult to visualize influenza nucleocapsids by the electron microscope (Compans and Dimmock, 1969; Bachi *et al.*, 1969), but evidence obtained from studies with paramyxoviruses, which have a larger single nucleocapsid, would suggest the next step in viral maturation is alignment of viral nucleocapsids beneath M (Compans *et al.*, 1966; Nagai *et al.*, 1976). It has even been proposed that M serves as a binding site for nucleocapsids (Nagai *et al.*, 1976).

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Virion formation occurs by the process of budding in which there is an out-folding of those areas of the plasma membrane containing the full complement of viral components. As the out-fold increases in size so the base constricts until

eventually the virion is released. In WSN infected CEF cells virus is released from both the free and adherent surfaces of the cell (Rodriguez Boulen and Sabatini, 1978). However, in some systems there is asymmetric budding with virus release occurring at only one of the cell surfaces. Thus in the chorioallantoic membrane of infected chicken embryos virus budding occurs only at the free luminal cell surfaces (Murphy and Bang, 1952), while in influenza infected MDCK cells, virus is released only from the free apical cell surfaces (Rodriguez Boulen and Sabatini, 1978; Roth et al., 1979).

10. Packaging of a segmented genome

The genome of influenza virus comprises at least 8 segments of RNA (see above). For a progeny virus particle to be infectious it must contain at least one copy of each segment. Two possibilities have been suggested as to how this might be achieved. The first is that viral RNA segments are incorporated randomly into virions from an intracellular pool. If on a random basis only 8 segments are incorporated into each virion then just 0.22% of the particles would contain the 8 different genes required for infectivity (Table 3). To achieve an infectivity to particle ratio of 10%, a value often observed for egg grown FP/R, each virion would have to package between 12 and 13 RNA segments (Table 3). As a consequence, virions would be expected to contain approximately 1.5 times the MW of the 8 segments. Sequence analysis has shown the combined MW of all 8 RNA segments to be approximately 4.8×10^6 daltons (Sleigh et al., 1979) whereas estimates of the amounts of RNA per virion range from 2.7×10^6 to 3.6×10^6 daltons (calculated from the data of: Ada and Perry, 1954; Frisch-Niggemeyer and Hoyle, 1956; Reimer et al., 1966). Therefore unless the calculations for the amounts of RNA per virion are underestimated it would appear that random incorporation is not the mechanism by which the genome of influenza is packaged.

Table 3

Probability of a virus particle obtaining a complete genome by random incorporation of RNA segments assuming that 8 different segments are essential for infectivity

Total RNA segments per virion	% of virions containing the 8 different segments
8	0.22
9	1.06
10	2.8
11	5.6
12	9.3
13	13.9
14	19.7

Probabilities were calculated using the form of the "Inclusion and Exclusion Law":

$$P = \sum_{v=0}^{n-1} \binom{N}{V} (-1)^{V} \left(1 - \frac{V}{N}\right)^{M}$$

where N = number of segments
M = number of segments selected

(Feller, 1968)

I am grateful to Prof. P. J. Harrison for advice concerning this problem.

It is however possible to modify the idea of random incorporation such that packaging of one segment restricts the packaging of subsequent segments (Prof. P. J. Harrison, personal communication). Such modifications need not be discussed here except to say that models, conforming to the observed infectivity to particle ratio, can be constructed in which only one copy of each segment need be packaged.

A second mechanism suggested for packaging of the influenza genome is that each viral RNA segment required for infectivity becomes linked to form a specific complex. This complex is then incorporated into the budding virion. There is evidence that viral RNP segments may be linked from electron microscope examinations of disrupted, or partially disrupted virions where what appears to be the RNP, is sometimes observed as a continuous strand some 6 nm long (Schulze, 1972; Almeida and Brand, 1975). However, no such continuous RNP strands have been observed in infected cells and any attempts to extract RNPs from cells yields RNPs of three (Duesberg, 1969; Pons, 1971) to five (Rees and Dimmock, unpublished data) size classes. Thus if linkage of RNPs is the mechanism for achieving a full genetic complement in progeny virions then such linkages must be somewhat fragile.

11. The Avian Erythrocyte

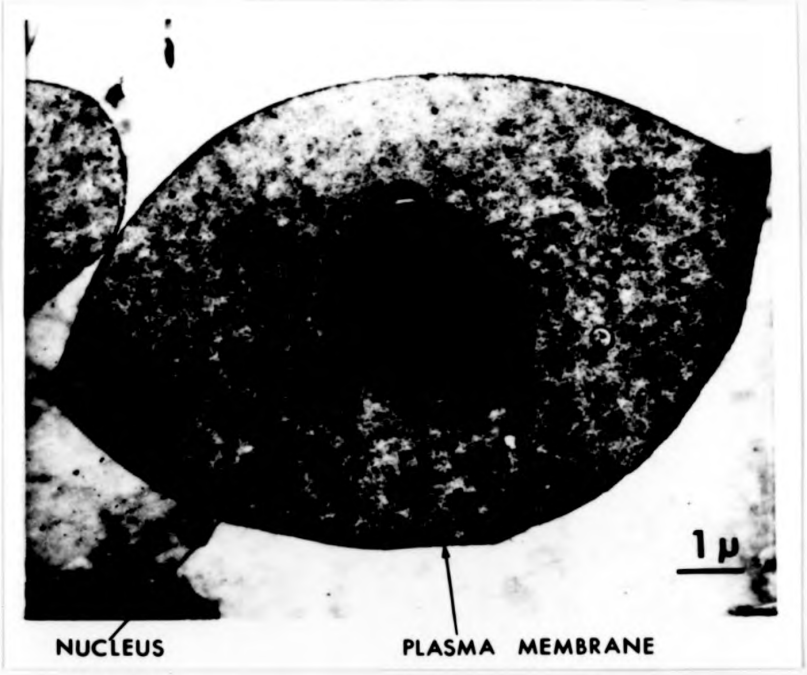
The avian erythrocyte in contrast to the doughnut-shaped, anucleate, mammalian erythrocyte is a nucleated, flattened ellipsoid (Fig. 2). These highly differentiated cells arise by a process termed erythropoiesis.

a) Erythropoiesis in the adult chicken

The stages of erythrocyte development are shown in Fig. 3. The erythroblasts, which are the precursors of erythrocytes, are located in the bone marrow (Lucas and Jamroz, 1961; Olson, 1963; Godet, 1974; Nigon and Godet, 1976). Here these cells divide and develop within sinuses lined by endothelial cells (Olson, 1963).

Erythroblasts are larger than cells of subsequent stages, contain mitochondria and are sometimes observed to have an amoeboid shape (Lucas and Jamroz, 1961). The nucleus is large in relation to the cytoplasm and stained preparations show it to consist of an open coarse network with chromatin that is clumped more than in other blast cells (Lucas and Jamroz, 1961). Clearly visible in these cells is a nucleolus. Erythroblasts are generally considered the only cells of the erythrocyte series which are capable of DNA replication and cell division (Lucas and Jamroz, 1961; Williams, 1972).

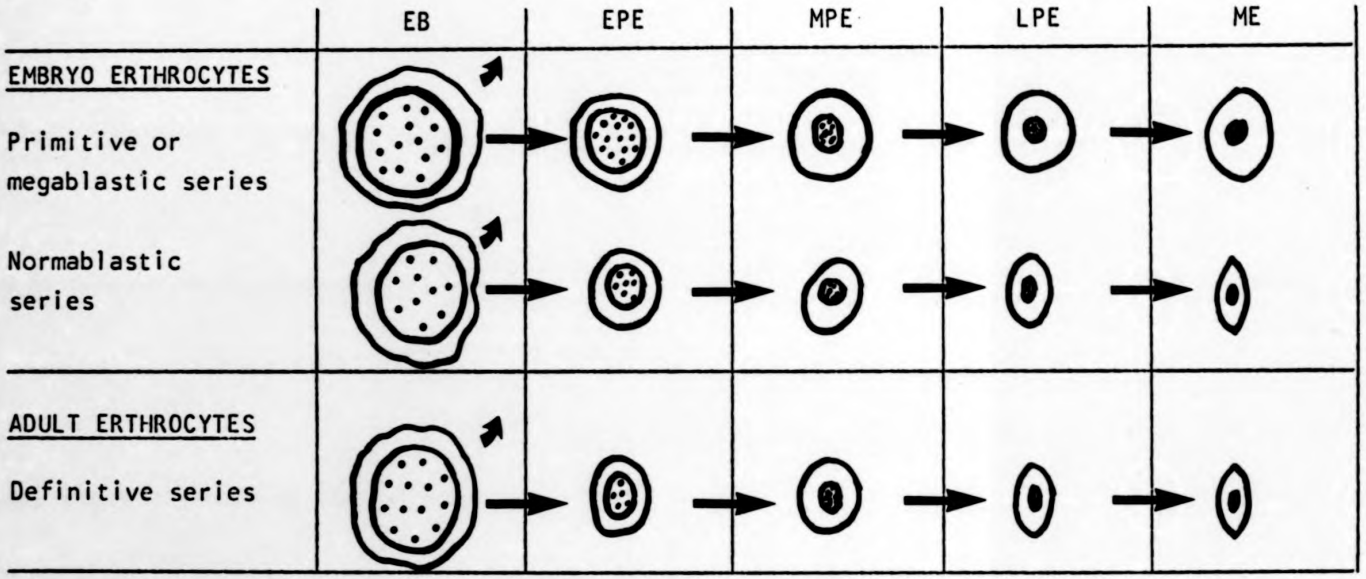
Fig. 2



Electron micrograph of an avian erythrocyte from a 13-day old chicken embryo.

(Courtesy of Miss J. Wignall).

Fig. 3



The Stages of Erythropoiesis in Chicken Embryos and in Adult Chickens

EB, Erythroblast; EPE, Early-Polychromatic Erythrocyte; MPE, Mid-Polychromatic Erythrocyte; LPE, Late-Polychromatic Erythrocyte; ME, Mature Erythrocyte

Erythroblasts give rise to early polychromatic erythrocytes. These cells differentiate through the stages of mid- and late-polychromatic erythrocytes into mature erythrocytes. The terms early-, mid- and late- refer to the blue, grey and orange phases of colour observed in the cytoplasm of stained preparations (Lucas and Jamroz, 1961).

Early-polychromatic erythrocytes are rounded cells with a homogeneous cytoplasm, lacking great numbers of mitochondria. Stained preparations show that the chromatin is clumped. The nucleolus is smaller than in the blast stage but still visible. Mid-polychromatic erythrocytes are rounded cells which are slightly smaller than the preceeding stage. No cytoplasmic mitochondrial spaces or nucleoli are visible. Furthermore, the nucleus of these cells is small relative to the cytoplasm. Late-polychromatic erythrocytes begin to show the transition from the round to the oval shape of the mature erythrocyte. In normal chickens all immature cells of the erythropoietic series are generally retained in the bone marrow. However, in chickens suffering from severe anaemia mid- and late-polychromatic erythrocytes are commonly detected in the blood stream (Lucas and Jamroz, 1961; Kabat and Attardi, 1967; Williams, 1972).

b) Erythropoiesis in the chicken embryo

Many of the experiments detailed in later sections involve the use of erythrocytes from either 13 day or 20 day chicken embryos. Therefore, a consideration of erythropoiesis in the chicken embryo is required.

Erythropoiesis in the chicken embryo is divided into primitive and normoblastic erythropoiesis. The former gives rise to a single generation of primary erythrocytes while the latter produces several generations of embryonic erythrocytes. These embryonic erythrocytes are morphologically distinct from primary erythrocytes. Also in their early developmental stages they are morphologically distinct from the definitive erythrocyte series of the adult bird (Lucas and Jamroz, 1961). Despite these differences the developmental stages of primitive, embryonic and adult erythrocytes are given a similar terminology (Fig. 3).

Primitive erythropoiesis (also called megaloblastic erythropoiesis as it yields large cells), starts in the area opaca, spreads to the area vasculosa and finally reaches the entire vitelline or yolk sac (Nigon and Godet, 1976). The first blood cells are detected 21 to 24 h after incubation. They have no haemoglobin and are located in structures called blood islands, i.e. small groups of cells enclosed by an endothelium. In these islands megaloblasts undergo extensive

mitosis (Lucas and Jamroz, 1961). However, although some haemoglobin synthesis can be detected during the second day, (Wilt, 1974) cytoplasmic and nuclear differentiation is inhibited until after 48 h incubation when the circulatory arc is completed (Lucas and Jamroz, 1961; Olson, 1963). Afterwards megaloblasts proceed to differentiate with a remarkable degree of uniformity. Thus, at 48 h practically all the cells are megaloblasts, but at 65 h these have all differentiated into early and mid-polychromatic erythrocytes of the primitive series. The number of these cells reaches a peak at 93 h after which they decline rapidly. Only a few late-polychromatic erythrocytes are present at 2.7 days; but these increase to become the dominant cell type at 4.9 days, and then decline rapidly until by 6.7 days they have almost vanished. At 4.2 days a few (less than 1% of the total) mature erythrocytes of the primitive series are detected. However, by 5.1 days these are the dominant cell-type. Mature erythrocytes of this first erythropoietic series are found in considerable numbers in the circulating blood (see Table 4) up to the 16th day of incubation (Lucas and Jamroz, 1961).

Normoblastic erythropoiesis starts in the yolk sac before megaloblastic erythropoiesis has ceased and reaches a maximum between the tenth and fifteenth days of incubation (Nigon and Godet, 1976). The erythrocytes it produces are

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Normoblastic erythropoiesis starts in the yolk sac before megaloblastic erythropoiesis has ceased and reaches a maximum between the tenth and fifteenth days of incubation (Nigon and Godet, 1976). The erythrocytes it produces are

smaller, less granular and more oval than their counterparts of the primitive series (Romanoff, 1960). The yolk sac is not the only organ to possess erythropoietic activity. Erythropoiesis has been observed in the spleen between the 8th and 14th day (Sandreuter, 1951) and for a brief period in the liver at the 8th or 9th day (Karrer, 1961). Erythropoiesis in the bone marrow, which is the sole organ of erythrocyte production in the adult bird, starts on the 12th day but only becomes intense from the 16th day onwards (Godet, 1974). Normablastic erythropoiesis never shows the synchrony observed in megaloblastic erythropoiesis.

From the third day onwards there is an increasing tendency for immature erythrocytes to be retained at the loci of their origin. Thus by the 8th or 9th day, mitotic figures are rarely seen in circulating blood (Romanoff, 1960). Consequently, the stages of differentiation seen in the blood from normablastic erythropoiesis are usually from mid-polychromatic erythrocytes to mature erythrocytes (Lucas and Jamroz, 1961).

Normablastic mid- and late-polychromatic erythrocytes can be detected from 5 days. Their number increases rapidly so that by 12 days they represent over 90% of blood cells. However, by 13 days this figure declines to about 10%. Mature embryonic erythrocytes do not appear until after 8.7 days.

Their number rises gradually at first and then more rapidly such that by 16 days they constitute 90% of blood cells. This proportion rises still further such that by the 20th day almost 100% of blood cells are mature embryonic erythrocytes (Lucas and Jamroz, 1961; Romanoff, 1960). Hatching is generally on the 21st day.

The percentages of the various forms of erythrocyte found in chick embryo blood are summarized in Table 4.

c) Structural and metabolic changes accompanying erythropoiesis

Apart from changes in shape, avian erythropoiesis leads to a progressive loss of ribosomes and the membranes of the endoplasmic reticulum and golgi apparatus. Mature erythrocytes possess very few of these structures (Grasso, 1973; Zentgraf et al., 1971). Another more obvious structural feature is a reduction in nuclear volume and an increase in condensation of chromatin (Lucas and Jamroz, 1961; Cameron and Prescott, 1963). Metabolically, erythropoiesis involves a transition from blast cells, active in macromolecular synthesis (DNA, RNA, protein) to mature erythrocytes with relatively little synthetic activity. This transition forms the basis of the following discussion.

Table 4

Percentages of various erythrocyte cell types in the circulating blood of the chick embryo

	Primitive series			Normablastic series		
Age of embryo (days)	Blast Cells	Polychromatic erythrocytes	Mature erythrocytes	Blast Cells	Polychromatic erythrocytes	Mature erythrocytes
2	100					
3	15	85				
3.9	3	93		2.6		
4.9		99		1.0		
5.1		9.9	79.5	10.0		
5.8			23.9	75.6		
6.1			20.3	38.0	40.6	
7.0			11.7	7.6	80.2	
8.0			10.3	0.2	89.5	
9.0			5.4	1.1	93.6	
10.0			6.6	0.5	92.5	
11.0			3.7	-	96.2	
12.0			3.8	1.0	95.2	
13.0			4.8	0.6	10.4	84.2
14.0			3.8	4.6	17.1	74.5
15.0			2.1	0.2	26.9	70.7
16.0			0.5	0.5	6.0	92.6
17.0			0.6	-	1.9	97.4
18.0			0.2	0.3	0.9	98.4

After Romanoff, 1960.

d) Structure and template activity of erythrocyte chromatin

The condensed chromatin of the mature avian erythrocyte has been shown to be a very poor template for bacterial RNA polymerase compared to chromatin from avian liver or kidney (Seligy and Muyagi, 1969), calf thymus (Tan and Muyagi, 1970), and avian erythroblasts (Gasaryan and Andreeva, 1972). In quantitative terms erythrocyte chromatin is only 1-2% as active as a template as deproteinized erythrocyte DNA (Seligy and Neelin, 1970). Removal of histones by acid extraction increases template activity to about 80% of deproteinized DNA. The remaining 20% can be accounted for by non-histone proteins (Seligy and Neelin, 1970).

That erythrocyte chromatin has a low template activity is also shown by treatment with DNAase II followed by isolation of Mg Cl_2-soluble and Mg Cl_2-insoluble fractions (Albrecht and Hemminki, 1977). Mg Cl_2-soluble, or template active, fractions have a high protein/DNA ratio and are enriched with nascent RNA compared to the template inactive Mg Cl_2-insoluble fractions (Bonner et al., 1975). Erythrocytes from 13 day embryos contain less than 30% of the amount of Mg Cl_2-soluble chromatin as brain, skin or muscle cells found in embryos of the same age (Albrecht and Hemminki, 1977). The template inactive regions of erythrocyte chromatin contain only small amounts of non-histone protein compared to the concentration of histones(Albrecht and Hemminki, 1977).

The mechanism for achieving condensation and template inactivation of erythrocyte chromatin is, at present, not understood. However, it is possible that protein fractions mediate in this process. The most abundant group of nuclear proteins the histones, become chemically modified during erythroycte maturation (Ruiz-Carrillo et al., 1974; Sung et al., 1977) and perhaps more significantly erythrocytes contain a histone (H5) which is tissue specific (Neelin, 1964; Hinlica, 1964). This histone replaces histone H1 (Hinlica, 1964). It is more basic than H1 and contains 37.1% lysines and arginines (Gavel et al., 1975) as opposed to 30% as in H1 (Bustin and Cole, 1968). Therefore, a possible model would be that synthesis of histone H5 at a particular stage in erythrocyte maturation would begin the process of chromatin condensation and that this would increase with the progressive synthesis and accumulation of the histone. However, this histone has been shown to be present in erythroblasts at about 70% of the level found in mature erythrocytes (Appels et al., 1972).

Some insight into the function of histone H5 might be gained by considering aspects of chromatin structure. The unit of chromatin structure is the nucleosome, a bead-like complex, which in higher eukaryotes is repeated approximately every 200 base pairs of DNA and covers most of the genome (Hewish and Burgoyne, 1973; Kornberg, 1974; Shaw et al., 1974;

Noll, 1974; Olins and Olins, 1974; Axel, 1975; Finch et al., 1975). Within the 200 base pair nucleosome repeat there are two operationally distinct regions: a nucleosome core containing 140 base pairs of DNA plus the histones H2a, H2b, H3 and H4; and a 60 base pair DNA linker with which histone H1 is associated (Shaw et al., 1976; Noll and Kornberg, 1977). Morris (1976) found that comparison of the fragments produced by brief digestion with micrococcal nuclease indicated that chicken erythrocyte chromatin had a longer nucleosome repeat (212 base pairs) than chicken liver chromatin (200 base pairs). More extensive digestion with micrococcal nuclease demonstrated that both tissues had 140 base pair nucleosome cores. The difference in nucleosome repeats is therefore due to an increase in the length of linker DNA, an increase that may be stabilized by the additional basicity of histone H5.

These results, together with similar studies on sea urchin sperm and gastrular cells (Spadofora et al., 1976), have led to the suggestion that genetic activity is correlated with the length of nucleosome repeat (Morris, 1976).

Non-histone proteins are unlikely to play an active role in erythrocyte chromatin condensation since they are reduced three-fold during maturation (Ruiz-Carrillo et al., 1974). There is however one protein (MW 50,000) which is apparently

selectively retained (Ruiz-Carrillo et al., 1974). Despite this reduction in the level of non-histone proteins several of them continue to be synthesized, albeit in small amounts, in mature erythrocytes (Jeter et al., 1976). The reason for this continued synthesis is unknown, although it is reasonable to speculate some involvement with transcriptional control.

e) Nucleic acid synthesis in avian erythrocytes

In view of preceding statements it is of no surprise that only small smounts of RNA synthesis can be detected in mature erythrocytes (Attardi et al., 1970; Madgwick et al., 1972; Maclean and Madgwick,1973; Zentgraf et al., 1975). This RNA is heterogenous in size, is capped (Attardi et al., 1970; Zentgraf et al., 1975) and binds to poly (U)-sepharose columns, indicating the presence of poly A tracks (Zentgraf et al., 1975). RNA synthesis in mature erythrocytes is inhibited by AMD and α-amanitin (Madgwick et al., 1972; Attardi et al., 1970; Zentgraf et al., 1975) suggesting involvement by RNA polymerase II (see below). However, this RNA synthesis exhibits several unusual properties compared to that in more active cell types. The majority of the RNA is retained in the nucleus (Attardi et al., 1970; Madgwick et al., 1972; Zentgraf et al., 1975) probably in the inter-chromatic regions (Zentgraf et al., 1975). In erythrocytes nearing maturity (i.e. late-polychromatic erythrocytes isolated

from anaemic hens) there are 4000 species of poly $(A)^+$ hn RNA present in the nucleus, but only about 100 species of poly $(A)^+$ mRNA in the cytoplasm (Laskey et al., 1978). Analysis of the kinetics of incorporation of radioactive precursors indicates that this RNA synthesis involves elongation of pre-existing chains (Zentgraf et al., 1975) with rapid subsequent degradation (Attardi et al., 1970; Zentgraf et al., 1975).

The pattern of nucleic acid synthesis during erythropoiesis has been studied using blood cells from ducks treated with phenlyhydrazine to induce anaemia. The different cells of the erythropoietic series were separated according to their buoyant density by centrifugation through bovine serum albumin (BSA) gradients (Attardi et al., 1970). The average buoyant density of both avian and mammalian erythrocytes increases with their chronological age and with their degree of maturity (Leif and Vinograd, 1964; Kabat and Attardi, 1967).

The bulk of RNA synthesis was found associated with erythroblasts and with the early- and mid-polychromatic erythrocytes. Late polychromatic and mature erythrocytes contributed only minor amounts (Attardi et al., 1970). Heterogenous RNA (hn RNA) synthesis was found to continue throughout erythrocyte development, although in decreasing amounts, while ribosomal

RNA (rRNA) synthesis ceased during the mid-polychromatic stage. However, the size distribution of hn RNA changed during maturation. Thus erythroblasts and early polychromatic erythrocytes produced large amounts of RNA with a sedimentation coefficient of 9S while the later stages produced very little. This 9S RNA is thought to be globin mRNA (Attardi et al., 1970) but was not translated in vitro.

f) Nucleotide polymerases in erythrocytes

Additional information about the metabolic capabilities of maturing and mature erythrocytes can be gained by studying relative nucleotide polymerase levels.

All three DNA dependent RNA polymerase enzymes are found in cells of the erythropoietic series (Longacre and Rutter, 1977; Krüger and Seifart, 1977). Although polymerase III, responsible for transcription of tRNA and 5S RNA genes (Weinman and Roeder, 1974) appears to be only just detectable even in erythroblasts (Longacre and Rutter, 1977).

Levels of RNA polymerase I, which synthesises rRNA, decrease rapidly during erythropoiesis such that early polychromatic erythrocytes contain less than half that found in erythroblasts. By the mature erythrocyte stage this enzyme is virtually undectable (Longacre and Rutter, 1977). By contrast

the levels of polymerase II, which synthesises mRNA decrease much more slowly. Early polychromatic erythrocytes contain 80% of the amount found in erythroblasts and significant amounts (15% of the erythroblast level) are still found in mature erythrocytes (Longacre and Rutter, 1977). Krüger and Seifart (1977) have confirmed the presence of significant amounts of polymerase II in mature erythrocytes.

Three forms of DNA polymerase are isolated from maturing erythrocytes. Two correspond to the α and β polymerases, which are generally found in eukaryotes, while the third form seems to be unique (Longacre and Rutter, 1977). DNA polymerases are present in significant amounts even in the late polychromatic erythrocyte stage (Longacre and Rutter, 1978). Williams (1971) reported that DNA synthesis ceased at the erythroblast stage, while Attardi *et al*. (1966) found some DNA synthesis in polychromatic erythrocytes. Therefore, it is not clear whether the DNA polymerases found at late stages of erythrocyte development serve any function.

Two RNA-dependent ribonucleotide transferases have been identified in developing erythrocytes. One has the properties of a terminal ribonadenyl-transferase (poly (A)-polymerase) and is found in erythrocytes at all developmental stages (Longacre and Rutter, 1977). This fact complements the finding by Zentgraf *et al*. (1975) that hn RNA of mature

erythrocytes contains poly A regions. The second ribonucleotide-transferase is capable of utilizing ribotriphosphates other than ATP (Longacre and Rutter, 1977). A similar activity has been found in other cell types (Wilkie and Smellie, 1968; Schafer et al., 1972; Niessing and Sekeris, 1973; Mikoshiba et al., 1974) although no specific function seems to have been ascribed to it. The levels of this enzyme are not significantly altered during the course of erythroid maturation until the final stage when a precipitious decline is observed (Longacre and Rutter, 1977). Its presence at normal levels when most other nucleotide polymerizing activities have declined suggests that it is very stable and/or has a continuing function in cellular metabolism.

From these results it is clear that the synthetic capability of erythrocytes steadily decreases during maturation. However, the final product of this maturation, the mature erythrocyte, is not completely metabolically inert. It is of interest to note that the shut down in nuclear activity during erythropoiesis is reversible. Introduction of the erythrocyte into the cytoplasm of a metabolically active cell results in expansion of erythrocyte chromatin followed by a resumption of nucleic acid synthesis (Harris, 1965, 1967; Bolund et al., 1969; Harris et al., 1969). This reactivation has been described as starting not from zero, but from the 'smouldering activity' already present with erythrocyte (Zentgraf et al., 1975).

12. Other cell types found in circulating avian blood

a) Thrombocytes

i) Thrombocytes in the adult

These are the smallest cells seen in the blood of the fowl. They vary considerably in size and form (Olson, 1963). The typical thrombocyte is oval with a round nucleus in the centre of a clear cytoplasm. There are two or three small, brightly red-staining granules at the pole of the cell. The chromatin of the nucleus is dense and is clumped into relatively coarse masses.

The definitive thrombocytes of the adult arise from thromboblasts located in the bone marrow. These differentiate into mature thrombocytes through the stages of early, mid and late immature thrombocytes (Lucas and Jamroz, 1961). In the adult, thrombocytes comprise approximately 0.8% of blood cells (Olson, 1963).

ii) Thrombocytes in the Embryo

The thrombocytes of the embryo appear as a clearly defined cell line soon after the primary erythroblasts are well established and are clearly present by 68 h incubation (Lucas and Jamroz, 1961).

Thrombocytes of the embryo are seldom ovoid in shape and show other differences from adult thrombocytes. For example, embryonic thrombocytes contain larger specific granules (Lucas and Jamroz, 1961). As a consequence of these differences a different terminology exists for cells of the embryonic thrombocyte series (Lucas and Jamroz, 1961). Thus thromboblasts give rise to large embryonic thrombocytes which give rise to medium embryonic thrombocytes and finally, to small embryonic thrombocytes.

Thromboblasts disappear from the circulation by about the 8th day of incubation (Lucas and Jamros, 1961). It is not until nearly hatching that definitive thrombocytes appear (Lucas and Jamroz, 1961).

b) Leukocytes

In the adult bird leukocytes account for about 0.6% of blood cells (Olson, 1963).

In the embryo leukocytes are seen only very rarely up until about the time of hatching (Lucas and Jamroz, 1961). In embryos 14 to 16 days old leucocytes form about 0.01% of blood cells. By 20 days this has risen to about 0.4% (Romanoff, 1960).

MATERIALS

1. Radiolabelled Components

These were obtained from the Radiochemical Centre, Amersham, Bucks., and had the following specific activities:

L-[4,5-^{3}H] Leucine, 40-60 Ci/mmol; L-[^{35}S] methionine, 600-800 Ci/mmol; Phosphorus-32, 80-130 Ci/mg of phosphorus; [methyl- ^{3}H] thymidine, 18-25 Ci/mmol; [5-^{3}H] uridine, 25-30 Ci/mmol.

2. Electrophoresis Components

Acrylamide and N-N'-methylene bisacrylamide (specially pure) were obtained from BDH Chemicals Ltd., Poole, Dorset. N,N,N',N'-tetramethylenediamine (TEMED) was obtained from Bio-Rad Laboratories Ltd., Richmond, California.

3. Chemicals

Bovine serum albumin (BSA) Fraction V and Ficoll (analytical grade) were obtained from Sigma London Chemical Co. Ltd., Poole, Dorset. Sheep anti-rabbit fluorescent immunoglobin was obtained from Wellcome Laboratories, Beckenham, Kent.

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Nonidet P40 (NP40) was obtained from BDH Chemicals Ltd., Poole, Dorset, and Tri-isopropylnaphalenesulfonic acid (TNS) was obtained from Eastman-Kodak, Rochester, New York.

4. Enzymes

Trypsin Type III and DNase Type I were obtained from Sigma London Chemical Co. Ltd., Poole, Dorset.

5. Tissue Culture Medium

All tissue culture media and newborn calf serum were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland.

METHODS

1. Breeds of Chicken

Two breeds of chicken were used in these experiments. One was a hybrid strain, obtained from Locksley Ltd., Nuneaton, which was derived from a Light Sussex female and a C020 male. This was used for experiments detailed in the first results section. However, the breeder went out of business and a new supplier was found (Hawksley Chicks Ltd., Evesham). The breed of chicken obtained from this supplier was White Legome. No significant differences in response to infection by viruses could be found between erythrocytes of the two breeds.

2. Cells

Avian erythrocytes were obtained from 13-day old chick embryos by cutting the allantoic blood vessels and allowing blood to drain into the allantoic fluid for 5-10 min. This fluid was removed from the egg and erythrocytes collected by centrifugation at 500 g for 10 min and washed twice in Alsevers solution (0.114 M glucose, 0.0718 M NaCl, 0.031 M Tri-sodium citrate, 0.00286 M citric acid). Erythrocytes from 20-day embryos were obtained by removing them from the egg, decapitating and allowing blood to drain into Alsevers solution. Erythrocytes from adult birds were withdrawn from a wing vein. Erythrocytes from both 20-day embryos and adult

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birds were washed twice in Alsevers solution after collection.

Primary chick embryo fibroblasts (CEF) cells were prepared as described by Morser et al. (1973). These were seeded at concentrations of 9×10^6 cells/3 mls in 5 cm plastic petri dishes. Cell monolayers were used when confluent.

3. Viruses

The type A influenza viruses used were the avian strain A/FPV/Rostock/34 (FP/R) (Hav 1 N1), A/FPV/Dutch/27 (Hav 1 Neq 1) and the human strains A/WSN; A/NWS, A/PR/8/34 (H0 N1); A/Jap/305/57 (H2 N2); A/HK/1/68 (H3 N2). All were grown by inoculation of about 10^5 PFU or EID50 into the allantoic cavity of embryonated chicken eggs. Those infected with avian strains were incubated for 18 h at 37°C and the others for 48 h at 33°C. Allantoic fluid was collected and used as the inoculum. Infectivity of avian strains were determined by plaque assay on CEF monolayers as described by Dimmock and Watson (1969) and Meier-Ewert and Dimmock (1970).

Newcastle Disease virus (NDV) strain Texas was grown in eggs as described for avian influenza strains except that infected eggs were incubated for 48 h at 37°C. Infectivity was determined by plaque assay on CEF monolayers.

Semliki Forest virus (SFV) was grown from an inoculum of infected mouse brain in a suspension of CEF cells (2.7×10^7 cells/ml) as described by Kennedy and Burke (1972). Infectivity was determined by titration on CEF monolayers.

4. Measurement of influenza virus haemagglutinin and neuraminidase

Haemagglutinin was measured using a 0.5% suspension of chicken erythrocytes (Borland and Mahy, 1968). Neuraminidase (NA) was assayed by the liberation of N-acetyl neuraminic acid from fetuin. This product can be converted to a coloured compound and thereby measured spectrometrically using reactions described by Warren (1959) and Aminoff (1961).

5. Preparation of labelled FP/R

[^{35}S] Methionine labelled FP/R for use as markers in polyacrylamide gel electrophoresis was prepared as described by Dimmock et al. (1977) in de-embryonated eggs. De-embryonated eggs were used as incorporation of the radioactive label into virion occurs with a greater effeciency (Dimmock et al., 1977).

6. Purification of erythrocytes by centrifugation through 10% Ficoll

A 10% solution of Ficoll was prepared in PBS and sterilized by autoclaving (5 lb psi for 10 min). Blood cells (2 ml) in Alsevers solution were diluted with ice cold 10% Ficoll (1 ml) to a final concentration of 4×10^8 cells/ml. This mixture (1.5 ml) was layered onto 10 ml of 10% Ficoll and centrifuged at 100 g for 10 min at $4^{\circ}C$. Erythrocytes sedimented to the bottom of the tube leaving a mixture of white cells and erythrocytes at the interface. The purity of these cell populations was monitored by phase contrast microscopy and by the use of a Coulter Channelyzer C-1000 (Coulter Electronics Ltd., Harpenden, Herts).

7. Analysis of blood cells by the use of a Coulter Channelyzer

3×10^6 Cells were added to 20 ml of Isoton II (Coulter Electronics Ltd., Harpenden, Herts) and pumped through a Coulter Channelyzer C-1000 (Coulter Electronics Ltd., Harpenden, Herts). This machine measures and displays in the form of a trace (see Section 1) the distribution of suspended particles as a function of their relative volumes. Calibration of the machine with standard sized sephadex beads and application of the appropriate formula (see below), allows conversion of relative volumes to actual volumes.

8. Calculation of actual cell volumes from the Coulter Channelyzer

Calculation of actual volume is achieved by using the following formula:

$$\left[\text{Channel No.} \times \frac{WW}{100} + BCT\right] \times TF = \text{Cubic microns}$$

The Coulter Channelyzer separates particle size into 100 channels. The channel number corresponds to the position of the peak on the trace. Base Channel Threshold (BCT) determines the smallest particle size to be included in the 100 channel size analysis.

Window Width (WW) determines the percentage of the 100 channels of the Coulter scale to be included in the analysis.

TF is a constant, the value of which is determined by calibration of the machine with standard sized sephadex beads.

9. Infection of cells

Erythrocytes in suspension (10^7 in 1 ml medium 199) and CEF monolayers were infected with influenza A virus at moi of about 30 PFU or EID50/cell. Erythrocytes and CEF monolayers were infected with SFV and NDV at 30 PFU/cell. Virus was adsorbed to cells for 60 min at 37°C, following which cells were washed twice with medium 199. After infection, cells were incubated in medium 199 (10^7 cells/ml) containing 5% newborn calf serum buffered with 20 mM HEPES-NaOH to pH 7.4. Erythrocytes were kept in suspension on a Rolamix mixing wheel (Luckham & Co. Ltd., Burgess Hill, Sussex). The period of infection was measured from the time that virus was added to cells.

10. Radiolabelling of cells with ^{35}S-methionine

Prior to labelling, cells (10^7 erythrocytes/ml or ~ 10^7 CEF cells in a 5 cm plastic petri dish) were washed twice with buffered Earles saline to remove all traces of maintenance medium. Labelling was carried out in 1 ml Earles saline buffered with 20 mM HEPES-NaOH to pH 7.4 and containing 100 μCi ^{35}S-methionine.

11. Dispersal of agglutinated erythrocytes following infection with influenza A viruses.

Aggregates of erythrocytes produced by infection with influenza A viruses were dispersed by incubating the cells for 30 min at 37°C in Earles saline containing 25 mM Tris-HCl pH 7.7 and 10 mg/ml trypsin (Type III). This treatment did not affect the ability of erythrocytes to synthesize labelled viral proteins as determined by polyacrylamide gel electrophoresis (see Section 1).

12. Polyacrylamide gel electrophoresis (PAGE)

20 cm x 20 cm Polyacrylamide gradient slab gels containing sodium dodecyl sulphate (SDS) were prepared using the Tris-glycine buffer system (Laemmli, 1970). The polyacrylamide gradient was from 10-30% w/v and this was stabilized by 0-8% glycerol. The reagents, along with their amounts, used in the preparation of these polyacrylamide gels are shown in Table 5.

All solutions of reagents were prepared using double distilled water. Gradients were prepared using a twin chambered gradient maker and poured under gravity. Ammonium persulphate concentrations were designed such that after

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Table 5

Reagents used in PAGE

Standard Solutions of Reagents	Concentration of Acrylamide			
	10%		30%	
	Vol in mixing Chamber (ml)	molar or % concentration	Vol in mixing Chamber (ml)	molar or % concentration
60% acrylamide plus 0.282% bis-acrylamide (ratio of bis to acrylamide is 1 : 212)	4.16	10%	12.50	30%
10% SDS	0.25	0.1%	0.25	0.1%
1 M Tris/HCl pH 8.6	9.3	0.37M	9.30	0.37M
H_2O	10.93		0.25	
80% Glycerol	-	-	2.50	8%
TEMED	0.033		0.033	
1% Ammonium persulphate	0.33	0.0132%	0.165	0.0066%

pouring acrylamide polymerisation started at the lowest concentration of acrylamide (i.e. the top of the gel) and proceeded downwards. This was to minimize disturbance to the gradient through heating effects.

Samples were prepared by heating in a boiling water bath for 2 min with 1/10th of a volume of 1% β-mercaptoethanol together with 1.4% w/v SDS. After electrophoresis at 20 mM for 13 h the gels were dried under suction onto a filter paper and exposed to Kodirex film (Kodak Ltd., Hemel Hempstead, Herts) for 5-8 days.

13. Determination of the amount of radioactivity in protein bands

Protein bands were cut out of dried polyacrylamide gels and placed into 3 ml of gel slice scintillant. This comprized of 4.2% NCS tissue solubilizer (Amersham, Arlington Heights, Illoinis), 4.6% liquifier (4 g 2-5-diphenyloxazole, PPO), 50 mg p-bis (2-(5-phenyloxazoly)-benzene, POPOP; 42 ml toluene), 90.2% toluene and 1% water. Slices were incubated for 48 h at 37°C and then cooled for 6 h at 4°C before counting in a Packard Tri-Carb scintillation counter. Values were normalized for differences in amounts of protein by comparing

the radioactivity in four equivalent areas (not containing viral proteins) of each sample track.

14. Radiolabelling of cells with ^{32}P

Erythrocytes and CEF cells were incubated for 1 h at 37°C in phosphate-free GMEM (GMEM-P) containing 5% newborn calf serum, which had been dialysed against Earles saline buffered to pH 7.5 with 10 mM Tris-HCl. Following this, the cells were transferred to GMEM-P containing 5% dialysed newborn calf serum and ^{32}P (1 mCi/10^8 cells). After a 2 h incubation period at 37°C, ^{32}P containing medium was removed and the cells infected with FP/R (dialysed to remove phosphates) as described previously. Once the period allowed for adsorption of virus was over, the ^{32}P containing medium was added back to the cells and incubation at 37°C continued for a further 7 h. After this time the cells were washed four times in ice-cold PBS and the RNA extracted.

15. Extraction of total cell RNA

Extraction of RNA was as described by Logan (1979). At the end of the labelling period, cells were washed three times in ice-cold PBS and then pelleted by centrifugation. Cell pellets were resuspended and dissolved in 50 mM Tris (pH 8.5) containing 1% (w/v) tri-isopropylnapthalene-sulphonic acid and 6% (w/v) 4-aminosalicyclic acid and deproteinized by addition of an equal volume of a phenol-chloroform-isoamyl alcohol mixture, (500 g of phenol (detached crystals), 70 ml of redistilled m-cresol and 0.5 g of 8-hydroxquinoline, saturated with 50 mM Tris, pH 8.5 (50 volumes); chloroform (50 volumes), and isoamyl alcohol (1 volume)). After vigorous shaking and centrifugation the aqueous phase was removed and extracted twice more with the phenol mixture, twice with chloroform-octanol (24:1 v/v) and finally, twice with ether. Residual ether was blown off in a stream of N_2, and NaCl was added to a final concentration of 100 mM. Most of the cell DNA was removed after gentle mixing with an equal volume of ethanol for 15 sec at room temperature, and centrifugation at 1000 g for 3 min to remove the precipitate. The remaining nucleic acid was precipitated by addition of a further 1.5 volumes of ethanol and storage overnight at -20°C. Yeast tRNA (4 mg/ml) was added to aid precipitation of nucleic acid.

The precipitated RNA was recovered by centrifugation (30,000 g) for 30 min at 4°C and dried in a stream of N_2. Contaminating

DNA was removed from this preparation by incubation with DNase (100 μg/ml) for 2 h at 37°C and the purified RNA re-extracted using phenol. RNA was analysed by PAGE using modifications, described by Crumpton et al. (1978) of methods developed by Floyd et al. (1974) and Palese and Schulman (1976).

16. Fluorescent antibody staining

a) Preparation of antisera against FP/R ribonucleoprotein (RNP)

Antisera was prepared by inoculation of purified RNPs into New Zealand white rabbits as described by Kelly and Dimmock (1974).

b) Fluorescent antibody staining of erythrocytes

Erythrocytes which had become aggregated following infection with FP/R were dispersed by treatment with trypsin (see Section 1). Residual inoculum virus was inactivated by resuspending the cells in PBS adjusted to pH 3 for 1 min at 4°C following which the pH was returned to 7.5 by the addition of PBS containing NaOH. Cells were then washed, resuspended in maintenance medium and incubated at 37°C for the required periods of time. On completion of incubation cells were washed, resuspended in maintenance medium containing 20%

newborn calf serum and smeared on a microscope slide. Smears were dried with a fan and fixed in acetone at 4°C. Cells were stained by the indirect technique, firstly with antisera against FP/R RNPs for 30 min at 37°C and then with Fluorescein isothiocyanate (FITC) conjugated sheep anti-rabbit IgG for the same period.

After incubation with serum, preparations were soaked in PBS for 40 min with six changes of solution to reduce the background level of fluorescence. Fluorescence was observed with a Reichart Binolux II microscope and all photographic procedures were kept constant to allow direct comparison of the prints.

c) Fluorescent antibody staining of CEF cells

CEF cells were grown on ethanol washed, heat-sterilized 16 mm glass coverslips in 5 cm plastic petri dishes. Plates were seeded at 1-2 x 10^6 cells per plate and allowed to grow to 30-50% confluency in maintenance medium. Infection and indirect fluorescent antibody staining of these cells was as described previously (Kelly and Dimmock, 1974; Minor and Dimmock, 1975).

17. TCA precipitation following radioactive labelling of cells

a) After radioactive labelling with precursors of protein synthesis

Cell samples lysed with 0.1% SDS were spotted onto 1 cm Whatman No.1 filter papers and immersed in 10% TCA containing 0.1% of the relevant cold amino acid. The filter papers were boiled for 15 min, washed twice in 5% TCA, twice in ethanol and once with ether. Filter papers were dried under an infra-red lamp and radioactivity determined with a Packard Tri-carb scintillation counter using toluene containing 2-5-diphenyloxzole (PPO) (5 g/litre) as scintillation fluid.

b) After radioactive labelling with precursors of nucleic acid synthesis

Cell samples lysed with 0.1% SDS were spotted onto 1 cm Whatman No.1 filter papers which were immersed in ice-cold 10% TCA for 30 min on ice. Following this, they were given two 10 min washes in ice-cold 5% TCA, two 10 min washes in ethanol and one wash in ether. Filter papers were dried and radioactivity determined as above.

c) TCA soluble radioactivity

This is the amount of radioactive label taken up by cells, but not incorporated into TCA precipitable material. It was determined by subtraction of TCA precipitable radioactivity from the total amount of radioactivity found in cells after the labelling period. The latter was determined by washing labelled cell samples 4 times in maintenance medium, resuspending in 100 µl of 10 mM Tris/HCl (pH 7.4) and digesting at room temperature for 24 h with an equal volume of soluene-350 (Radiochemical Centre, Amersham, Berkshire). Radioactivity was measured in a Packard tri-carb scintillation counter using toluene containing triton X-100 (33.3% v/v) and PPO (4.0 g/l) as scintillation fluid.

TCA precipitable ^{3}H-leucine was determined as follows: after labelling cells were washed three times with PBS, following which 4 volumes of 10% TCA (containing 0.05% w/v leucine) were added. This caused all erythrocytes to lyse. Cell extracts were incubated at room temperature for 2 h and precipitates collected by centrifugation (9900 g for 5 sec using an Eppendorf microfuge). The pellet was washed twice with 10% TCA, twice with ethanol, once with ether and dried overnight in a vacuum desicator. The pellet was resuspended in 100 µl of 10 mM Tris-HCl pH 7.5 and 100 µl of soluene-350 (Packard Instruments Ltd., Caversham, Berkshire) and incubated for 24 h at room temperature. Determination of radioactive counts was as described for total counts.

TCA precipitable ^{3}H-uridine was determined in the same way except that 4 volumes of ice-cold 10% TCA were added to erythrocyte samples and the nucleic acids allowed to precipitate for 30 min on ice. Nucleic acids were pelleted by centrifugation (9900 g for 5 sec using an Eppendorf microfuge) and the pellets washed twice in ice cold 5% TCA before being washed in ethanol and ether.

18. BSA gradients

a) Unbuffered balanced salt solution (UBSS)

This was prepared to be isotonic with chicken serum (Shortman, 1970). It contained 0.168 M NaCl, 121 vol; 0.168 M KCl, 4 vol; 0.112 M $CaCl_2$, 3 vol; 0.168 M K H_2 PO_4, 1 vol; 0-168 M Mg SO_4, 1 vol. Problems of erythrocyte clumping are reduced if gradients are centrifuged at pH 5.1 (Shortman, 1970). This is the pH of a solution of BSA. For this reason no buffering system was introduced into the balanced salt-solution.

b) Dialysis of BSA

A 15-20% solution of BSA Fraction V was prepared in deionised, distilled water and dialysed for 72 h at 4°C against 6 changes

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b) Dialysis of BSA

A 15-20% solution of BSA Fraction V was prepared in deionised, distilled water and dialysed for 72 h at 4°C against 6 changes

of deionised distilled water. To prevent bacterial growth six drops of chloroform were added to each change of dialysis water. After dialysis albumin was freeze dried and stored at -20°C.

c) Preparation of a 35% w/w stock solution of BSA

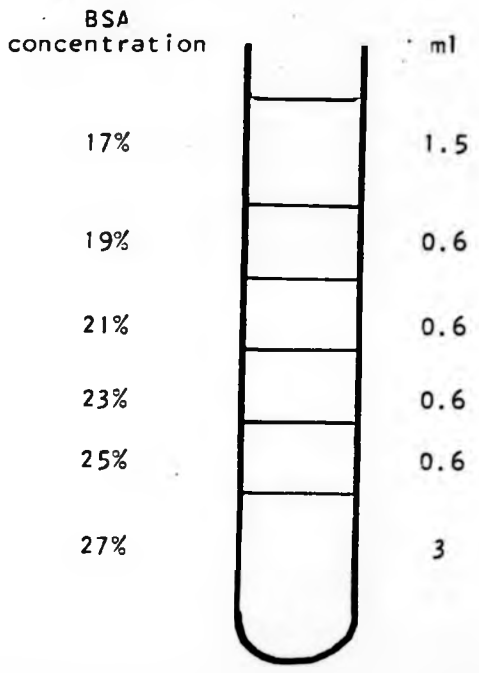
100 g Of dialysed, freeze dried, BSA was disolved in 181 ml of UBSS and 5 ml of deionised, distilled water. The water was to compensate for the slight osmotic pressure contribution of the albumin itself (Shortman, 1970). The concentration of this stock solution was checked by measuring its refractive index. A 35% w/w solution of BSA has a refractive index of 1.4003.

d) Preparation of BSA gradients

Discontinuous BSA gradients were prepared in 14 ml polycarbonate centrifuge tubes (MSE Scientific Instruments Ltd., Crawley, Sussex). The concentrations of BSA used were 27%, 25%, 23%, 21%, 19% and 17% (see Fig. 4). This figure shows the volumes of the various concentrations used in the preparation of a discontinuous BSA gradient. These concentrations were prepared by dilution of the BSA stock solution with UBSS. The accuracy of these dilutions were checked by refractometry. All gradients were used within 30 min of pouring. Blood cells

Fig. 4

Preparation of BSA Gradients



10^9 (the maximum number of cells that can be separated in a gradient of the type described is 1.5-2 x 10^9 cells, Dicke, 1970) were suspended in 1.5 ml of 17% BSA solution and gently pipetted on top of the 19% BSA layer. The gradient was centrifuged (1000g) for 30 min at 10^oC in a Sorvall RC-5B centrifuge using a HB4 swing-out rotor adapted for 14 ml tubes.

After centrifugation distinct layers of cells were visible in the gradient near the density interfaces. These layers were collected with the aid of a Pasteur pipette. The cell fractions between the 17 and 19% BSA layers and the 19 and 21% BSA layers contained few cells and so these were counted as one fraction, designated fraction 1. Fraction 2 was the cell layer between 21 and 23% BSA layers, fraction 3 was between 23 and 25% BSA layers, fraction 4 was between 25 and 27% BSA layers and fraction 5 consisted of those cells which formed a pellet at the bottom of the gradient. After each fraction had been collected it was diluted with maintenance medium, centrifuged, washed twice in maintenance medium and the number of cells counted with a haemocytometer.

19. Fractionation of Nuclei and Cytoplasm

For CEF cells this was achieved using the 'nuclear monolayer method' described by Hudson and Dimmock, 1977.

This technique was adapted for erythrocytes as follows. 10^7 Erythrocytes were pelleted (500 g for 10 min), maintenance medium removed and resuspended in 0.5 ml of nuclear monolayer buffer (NMB) (0.25 m sucrose, 1 mM $MgCl_2$, 10 mM Tris/HCl pH 7.4) containing 2% NP40 for 5 min at 0-4°C. This time was the same as that used for CEF cells although it was noted that all erythrocytes lysed within 1 min of addition of NP40 solution. Nuclei were pelleted and the NP40 solution removed and stored on ice as a cytoplasmic fraction. Treatment with NP40 solution was repeated, the nuclei resuspended in 50 µl NMB and their purity checked using phase contrast microscopy. The nuclei were stored at -70°C until analysis by PAGE.

The proteins in the cytoplasmic fraction were precipitated by the addition of 4 volumes of ice-cold acetone for 15 min at 4°C. The precipitated protein was collected by centrifugation (1000 g for 5 min). Residual acetone was removed by drying overnight in a vacuum desicator and pellets were resuspended in 50 µl of NMB using a soni-bath and stored at -70°C until analysis by PAGE.

Results & Discussion

Section 1

Infection of avian erythrocytes with Fowl Plague virus, Newcastle Disease virus and Semliki Forest virus

1. Introduction

Earlier work with heterokaryons produced by fusion of avian erythrocytes with 'enucleated' BHK cells (Kelly and Dimmock, 1974; Minor and Dimmock, 1976) had indicated that the avian erythrocyte nucleus may support the synthesis of some but not all influenza virus proteins. To investigate this possibility further erythrocytes were infected directly with the avian influenza strain FP/R. The erythrocytes used in the heterokaryon work were from either 11 day (Minor and Dimmock, 1976) or 13 day (Kelly and Dimmock, 1974) old chicken embryos. To be consistent with this data the present studies were also began with erythrocytes from chicken embryos. However, erythrocytes from 13 day old embryos were chosen since they are reported to contain a higher proportion of mature erythrocytes than the erythrocyte population of 11 day old embryos (Romanoff, 1960). Therefore results detailed in this section are from experiments with erythrocytes from 13 day old embryos unless stated otherwise.

Avian erythrocytes were also infected with two other viruses: Newcastle disease virus and Semliki Forest virus. These viruses have no absolute requirement for a functional host cell nucleus and so (unlike as is possible for influenza) their replication should not be directly affected by the comparatively dormant state of the erythrocyte nucleus.

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Therefore, these viruses may provide some measure as to whether the differentiated avian erythrocyte retains sufficient capacity (i.e. numbers of ribosomes, amounts of tRNAs, energy supply, etc.) for the synthesis of progeny virions.

2. Results

Preparation of purified erythrocytes

Preliminary experiments showed that newly synthesized radiolabelled viral protein could be detected in washed but unfractionated 13 day old chicken embryo blood cells infected with FP/R. However, 13 day embryo blood contained a small proportion (approximately 0.01%) of leucocytes (Romanoff, 1960). Therefore it was necessary to separate erythrocytes and leucocytes to show which were responsible for viral protein synthesis. These cells were separated by centrifugation through 10% Ficoll as outlined in Materials and Methods. The leucocyte fraction was further processed by a second centrifugation through 10% Ficoll. In this way two populations of cells were obtained: purified erythrocytes and an 'enriched' population of leucocytes consisting of approximately equal proportions of leucocytes and erythrocytes (i.e. a 10^4-fold enrichment of leucocytes). Blood from sixty embryos yielded approximately 4×10^6 leucocytes. The purity of these cell populations was monitored by phase contrast microscopy and by the use of a Coulter Channelyzer C-100 (Coulter Electronics Ltd., Harpenden, Herts) which measures the distribution of suspended particles as a function of their volume. Figure 5 shows that erythrocytes and leucocytes from the interface of the Ficoll separation consist of two

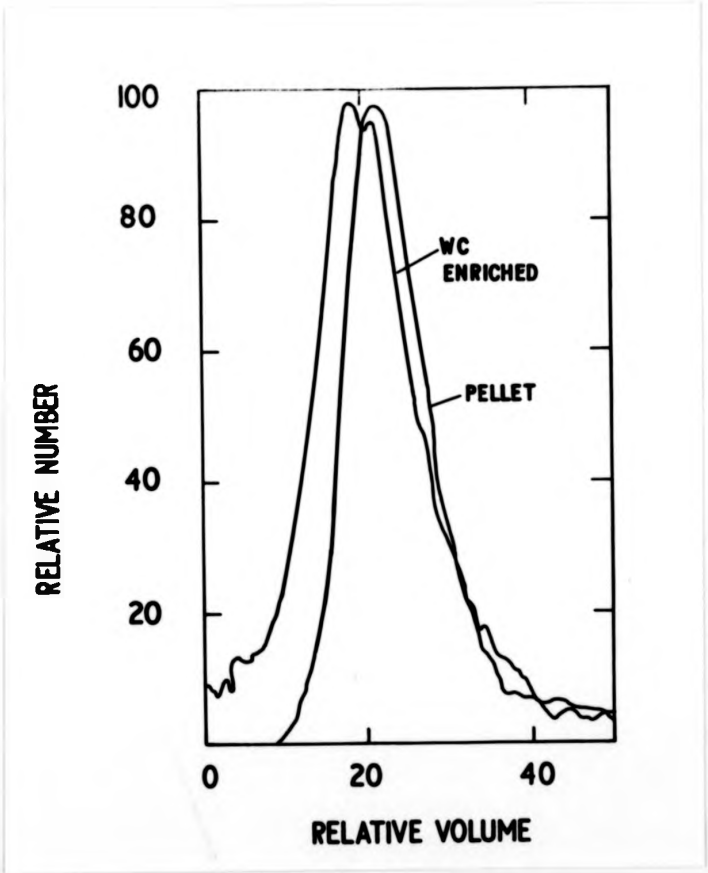

100
80
60
40
20
RELATIVE NUMBER
WC
ENRICHED
PELLET
0
20
40
RELATIVE VOLUME

Fig. 5

Size distribution of purified erythrocytes and of a preparation enriched for leucocytes (w.c.) made by centrifugation through 10% Ficoll

Cell volume is plotted against relative cell number.
The graph is a direct trace from a Coulter Channelyzer.

size classes. By applying the appropriate formula (see Materials and Methods) to the relative volumes, the actual volumes of the two size classes of cells were calculated as approximately 79 and 84 μm^3. Cells which pelleted through the 10% Ficoll were distributed in a single peak corresponding to a volume of 84 μm^3. These data are consistent with microscopic observation showing that interface material consists of leucocytes and erythrocytes, while pelleted material comprised only erythrocytes. The volumes given above are in agreement with values calculated from published figures (Romanoff, 1960). Therefore, centrifugation through 10% Ficoll offers a procedure for removing leucocytes from chicken blood.

Synthesis of proteins in erythrocytes and leucocytes infected with FP/R

Unfractionated blood cells, purified erythrocytes and cells from the enriched leucocyte population were infected with FP/R, radiolabelled and newly synthesized proteins analyzed by PAGE. Unfractionated blood and the purified erythrocyte preparation synthesized viral proteins in approximately equal amounts (Fig. 6). However, no significant protein synthesis was detected in the leucocyte preparation even on exposure of the autoradiogram for 8 weeks. Leucocyte viability as

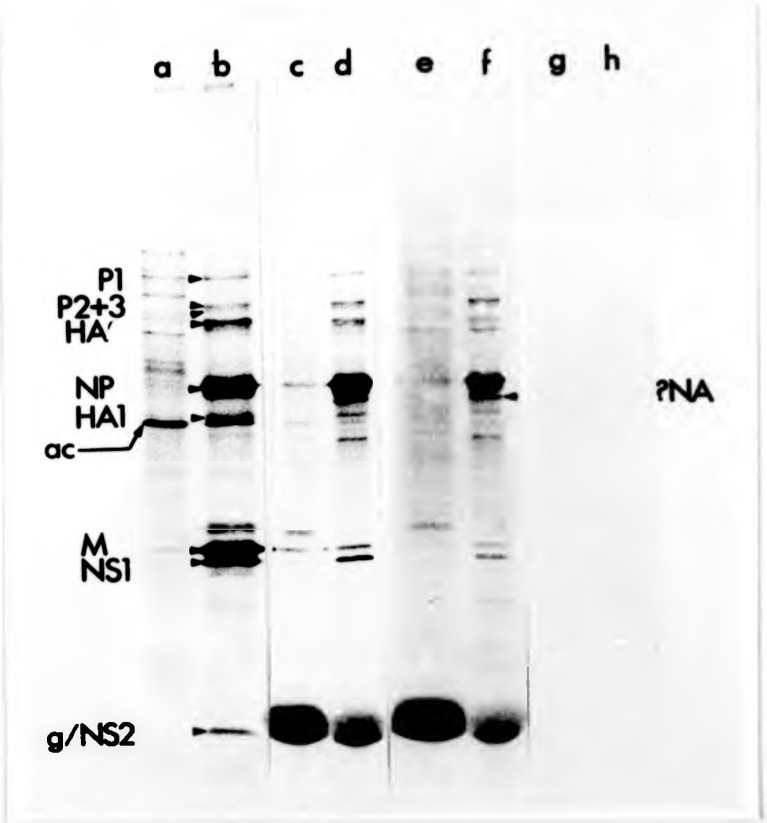

a
b
c
d
e
f
g
h
P1
P2+3
HA'
NP
HA1
ac
M
NS1
g/NS2
?NA

Fig. 6

PAGE of proteins synthesized in chicken cells infected with FP/R

a, b. CEF cells; c, d. unfractionated blood cells (10^7/ml); e, f. purified erythrocytes (10^7/ml); g, h. enriched leucocytes (2×10^6/ml).

a, c, e, g, were not infected and b, d, f, h were infected with 30 PFU/cell. Cells were radiolabelled with ^{35}S-methionine (100 $_u$Ci/10^7 cells) from 5 to 6 h p.i. Host proteins actin (ac) and globin (g) are also indicated. Nomenclature of FP/R proteins follows the established convention (Kilbourne et al., 1972; Lamb and Choppin, 1976; Inglis et al., 1976). Tracks c to f were loaded with approximately equal radioactivity (80,000 cpm) and g, h each with 8,000 cpm, the maximum available.

judged by exclusion of trypan blue was around 50% of the population. Since the enriched leucocyte population contained about 10^4 fold more leucocytes than whole blood it is concluded that these cells were not contributing significantly to the synthesis of viral proteins observed in infected blood. Although it was not necessary to purify erythrocytes for the following experiments this procedure was continued as an added precaution.

In this particular experiment, FP/R infected erythrocytes were observed to synthesize viral proteins with MWs corresponding to P1, P2, P3, uncleaved HA, NP, M and NS1. A protein band corresponding to HA1 could also be detected but not one corresponding to HA2. In addition, there was a protein migrating in advance of NP which appeared neither in uninfected erythrocytes or in CEF cells. This protein could be a non- or a partially glycosylated form of NA, which in its glycosylated form has a tendency, depending on the gel system, to co-migrate with NP (Skehel, 1972; Minor and Dimmock, 1975; Lamb and Choppin, 1976; Inglis and Mahy, 1979; Carver and Dimmock, unpublished data). Alternatively, this protein may be a degradation product of some larger polypeptide or a cellular protein whose synthesis was stimulated by infection.

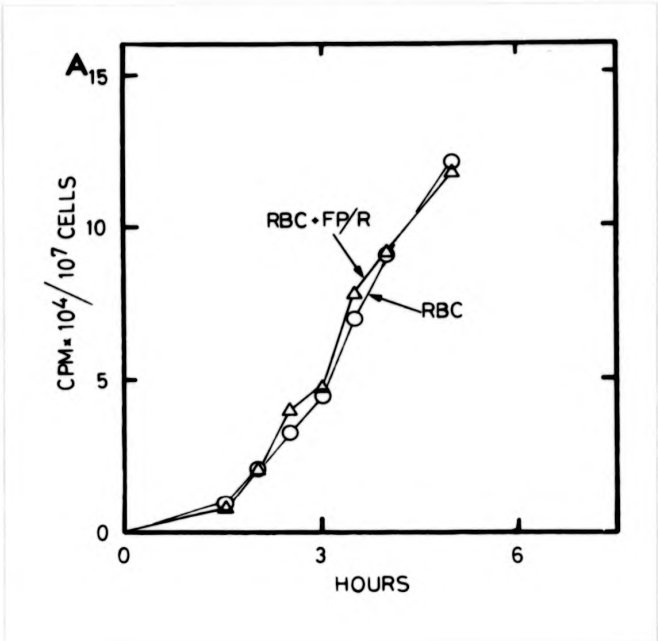
Comparison of the amounts of FP/R protein synthesized in purified erythrocytes and CEF cells

Equal amounts of protein (as determined by the method of Lowry et al., 1951) from infected erythrocytes and infected CEF cells were analysed by PAGE. Both cell types were infected with 30 PFU/cell and labelled from 5 to 6 h p.i. with ^{35}S-methionine (100 μCi/10^7 cells). A comparison made at this time was justified as the time courses of viral protein synthesis in erythrocytes and CEFs were found to be very similar (see Section 2). The autoradiogram of the gel was scanned with a Joyce-Loebl densitometer (the exposure of the autoradiogram used was calibrated to be within the linear response range of the film) and the area under the viral NP peak produced by each cell type was calculated and normalised for total protein. On this basis erythrocytes synthesized 5% of the amount of NP found in CEF cells, but if the calculations were made on the amount of NP synthesized per cell, then erythrocytes make 0.65% NP of that in CEF cells.

Effect of FP/R infection on protein and RNA synthesis in erythrocytes from 13 day old chick embryos

The introduction of self-replicating genetic information, i.e. FP/R into erythrocytes could conceivably stimulate metabolic activity within these cells. Metabolic activity in terms of RNA and protein synthesis was measured by incorporation of either ^{3}H-leucine or ^{3}H-uridine into TCA precipitable material. FP/R infection did not alter the total amount of ^{3}H-leucine incorporated by erythrocytes (Fig. 7a). However, as will be shown later (see Section 2) FP/R infection does reduce the synthesis of certain (notably globin) erythrocyte proteins.

Incorporation of ^{3}H-uridine was slightly increased in FP/R infected erythrocytes, at least until about 4 h p.i. (Fig. 7b). Whether such an increase was due to synthesis of viral or of host RNA cannot be determined simply on the basis of total counts. In an attempt to determine what sorts of RNA were being synthesised, in both infected and uninfected cells, erythrocytes were radiolabelled with ^{32}P, and their RNA extracted (see Materials and Methods) and analysed by PAGE. However, Fig. 8 shows that this approach was of limited value since only trace amounts of 28S and 18S ribosomal RNA could be detected. However, infection with FP/R did seem to reduce amounts of ribosomal RNA synthesized by erythrocytes from 13 day old chicken embryos.


A
15
10
5
0
CPM×10⁴/10⁷ CELLS
RBC+FP/R
RBC
0
3
6
HOURS

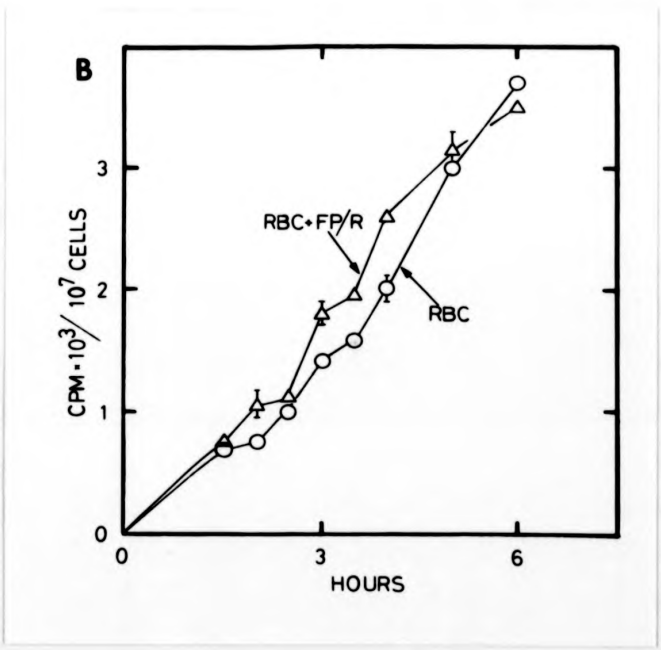

B
3
2
1
0
CPM·10³/10⁷ CELLS
RBC+FP/R
RBC
0
3
6
HOURS

Fig. 7a

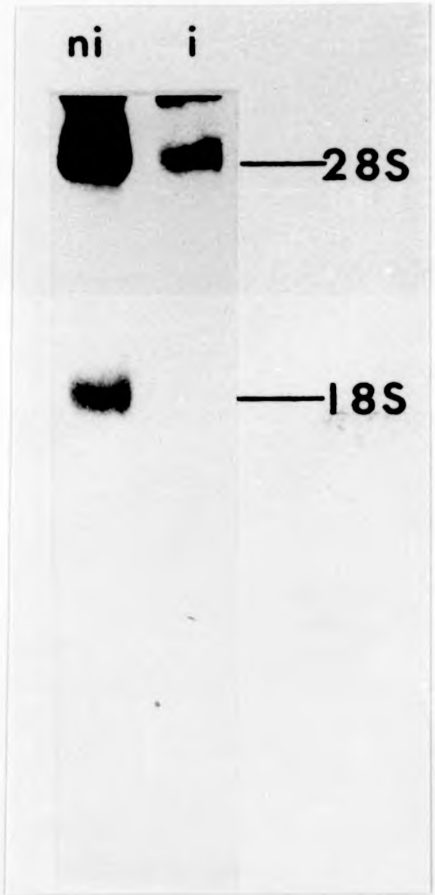
Incorporation of TCA precipitable ^{3}H-leucine in FP/R infected and non-infected erythrocytes

FP/R infected (30 PFU/cell) and mock infected erythrocytes were incubated for 1 h at 37^{o}C in medium 199 plus 10% newborn calf serum. Cells were then washed twice in Earle's saline/20 mM HEPES/1% 199 and resuspended in the same medium containing ^{3}H-leucine (10 $_{u}$Ci/10^{7} cells). Samples were taken at intervals and TCA precipitable radioactivity determined as described in Materials and Methods. Time zero refers to the time of addition of label.

Fig. 7b

Incorporation of TCA precipitable ^{3}H-uridine in FP/R infected and non-infected erythrocytes

Erythrocytes were treated as described in Fig. 7a but radiolabelled with ^{3}H-uridine (20 $_{u}$Ci/10^{7} cells).


ni
i
28S
18S

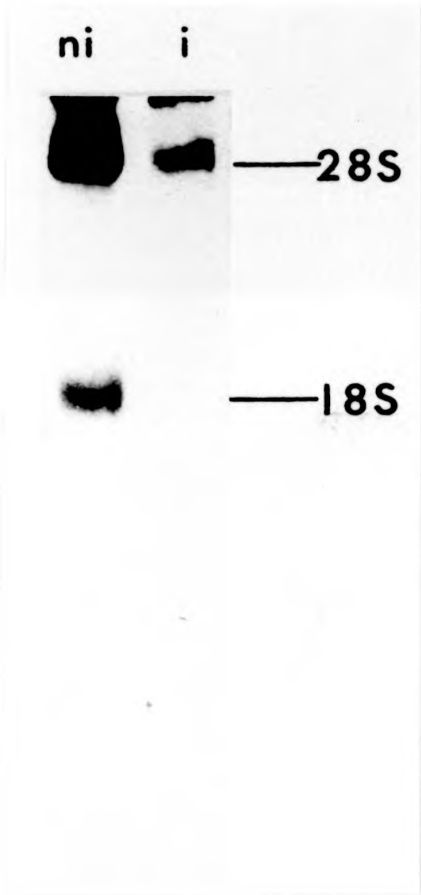

ni
i
28S
18S

Fig. 8

PAGE of RNA from FP/R infected and non-infected erythrocytes

RNA from infected (i) and non-infected (ni) erythrocytes analysed by PAGE. RNA from CEF cells was included as a marker.

Separation of agglutinated erythrocytes

It was no surprise that erythrocytes agglutinated upon infection with FP/R. Such cells retained up to 13% of the infectivity and up to 20% of the HA titre of the inoculum, even after extensive changes of medium and a 1 min incubation at $4^{o}C$ in PBS adjusted to pH 3 (pH 3 incubation will rapidly inactivate free virus (Stephenson et al., 1978)).

The problems caused by the retention of inoculum virus are exemplified in Fig. 9. Here the typical growth curve of FP/R in CEF cells is compared with the results of a similar assay with FP/R infected erythrocytes. With infected erythrocytes there was a gradual increase, from an initial value of 7.2×10^{6} PFU/cells, of 5-fold from 2 to 6 h p.i.; following which infectivity declined by 2.6 fold. This can be contrasted with an increase of 10,000-fold from 4 to 8 h p.i. in FP/R infected CEFs.

To determine whether the gradual increase in infectivity in FP/R infected erythrocytes was due to synthesis of progeny virions or to retention of inoculum virus, it was necessary to reduce the level of inoculum virus and as a prerequisite for this to separate the agglutinated erythrocytes.

Both aims were achieved by incubating FP/R infected erythrocytes with trypsin (Fig. 10) followed by an incubation in PBS adjusted

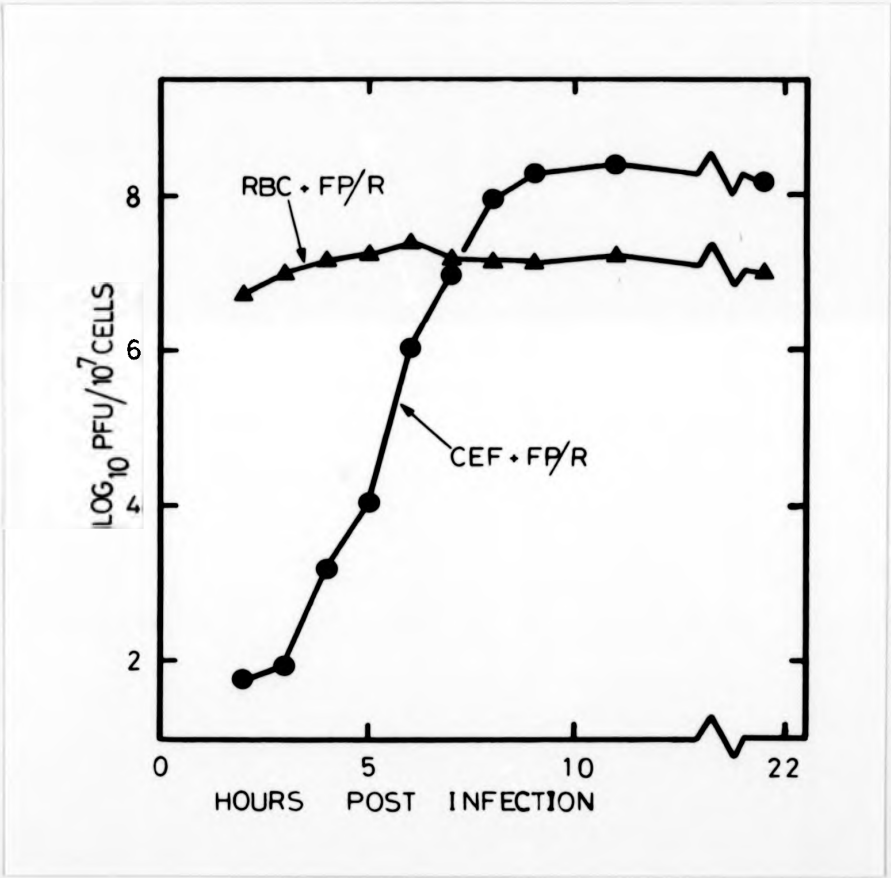
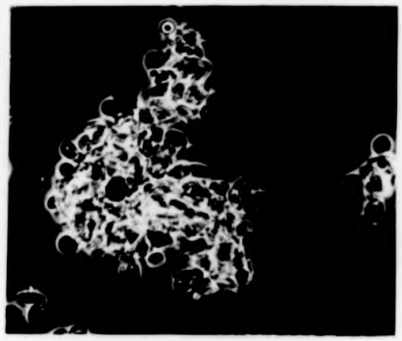

RBC + FP/R
CEF + FP/R
8
6
4
2
LOG_{10} PFU/10^7 CELLS
0
5
10
22
HOURS POST INFECTION

Fig. 9

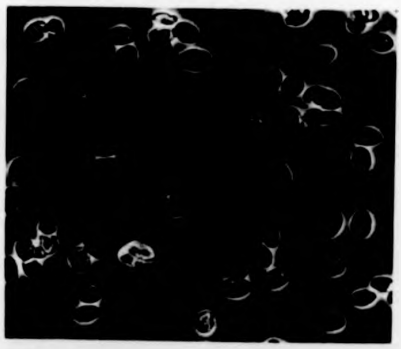
Comparison of the amounts of infectious virus associated with erythrocytes and CEF cells following infection with FP/R

CEF monolayers (approximately 10^7 cells/5 cm dish) and a suspension of erythrocytes (10^7 cells/ml) were infected with FP/R, (30 PFU/cell) incubated for 1 h at 37°C and washed for 1 min in PBS adjusted to pH 3. Duplicate samples each of 10^7 cells from both cell types were taken at intervals after infection, sonicated and assayed for infectivity on CEF monolayers.

A



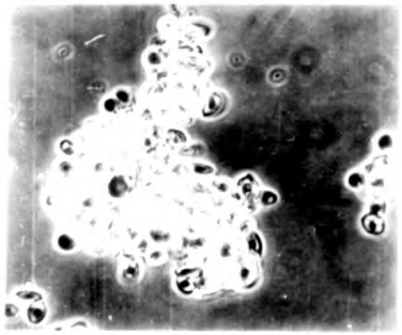
B



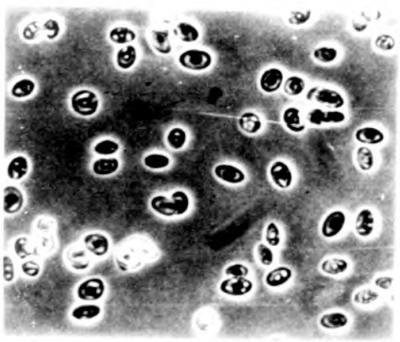
20μ

C

A



B



20μ

C

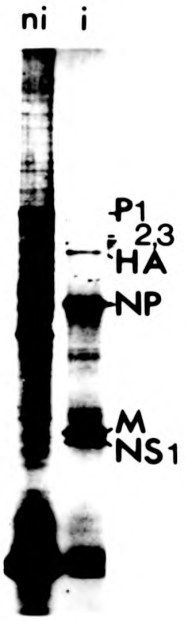


Fig. 10

Effect of trypsin on erythrocytes agglutinated by FP/R

A. Phase contrast micrograph of agglutinated erythrocytes 2 h after infection with FP/R.

B. Phase contrast micrograph of infected erythrocytes following treatment with trypsin (10 mg /10^7 cells).

C. PAGE of proteins synthesized by trypsin treated infected (i) and non-infected (ni) erythrocytes. Cells were radiolabelled with ^{35}S-methionine (100 μCi/ 10^7 cells) 5 to 6 h p.i.

to pH 3 (Materials and Methods). This reduced the PFU of the inoculum virus by 10^4 fold. Although up to 30% of the cells were lost after treatment with trypsin, cells remaining incorporated ^{35}S-methionine and synthesized the same amount of viral protein per cell as untreated controls (Fig. 10c).

Failure of FP/R to multiply in 13 day old embryo erythrocytes

After 1 h at 37°C the infected erythrocytes were washed, disaggregated with trypsin and treated with pH 3 buffer solution to reduce the level of input virus

Cells and tissue culture fluids were disrupted at intervals after infection and assayed on CEF monolayers. No rise in the amount of infectious virus present was detected (Fig. 14). Titrations were repeated in suspension in the presence of trypsin to enhance infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Again the level of infectious virus present did not rise above the initial level of 900 PFU/10^7 cells even though A/PR/8/34, which requires trypsin for plaquing was assayed successfully (Table 6). By calculating the ratio of NP synthesized in CEF cells : NP synthesized in erythrocytes, and knowing the number of PFU synthesized in CEF cells it was estimated that the infectivity in erythrocytes was over 400-fold less than the expected value (Table 7).

Table 6

Cell suspension plaque assay for the multiplication of FP/R in erythrocytes[a]

FP/R in Erythrocytes Postinfection (h)	+ Trypsin	- Trypsin
2.5	900[b]	nt
3.5	600	nt
8.0	400	nt
12.0	< 150	nt
24.0	< 150	< 150
A/FP/Rostock[c]	8.9×10^8	8.0×10^8
A/PR/8/34[c]	3.1×10^7	$< 10^2$

a. Samples of erythrocytes were mixed with 2.5 ml of 3×10^7 CEF cells/ml in suspension and dispersed in an equal volume of double strength overlay medium containing 100 µg/ml DEAE dextran and 20 µg/ml trypsin. FP/R and PR/8 were inoculated directly into the CEF suspension.

b. PFU/10^7 erythrocytes

c. PFU/ml of virus suspension

nt not tested

Table 7

Synthesis of PFU, haemagglutinating and neuraminidase activities in FP/R-infected erythrocytes[a]

	CEF	Erythrocytes		
		Expected	Observed	Expected/Observed
PFU	8.5×10^{6}[b]	6×10^{4}	$< 1.5 \times 10^{2}$	400
NA	17.0[c]	0.11	0.14[d]	0.8
HA	2000.0	13.00	< 0.6	> 21.7

a. Expected values were calculated from the ratio of NP synthesized in CEF cells and erythrocytes, i.e. 100 : 0.65 arbitary units (see Results)

b. All figures are for 10^{7} cells + culture fluids

c. OD_{549}/h

d. OD_{549}/h calculated from a 30 h incubation

Haemagglutinin and neuraminidase activity in FP/R-infected erythrocytes

Erythrocytes were infected and the levels of inoculum virus reduced as described above. Samples of cells were removed at intervals and disrupted by ultrasonication on ice. No haemagglutinating activity was detected. Standard NA assays incubated for 1 h at 37°C failed to give a positive result so incubation of the assay was continued for 30 h. Low but significant levels of neuraminidase activity were detected which increased with the duration of infection (Fig. 11). This neuraminidase was serologically identical with that of the FP/R (Wignall and Dimmock, unpublished data). It was calculated that the ratio of NP : neuraminidase activity synthesized in erythrocytes was similar to that in CEF cells (Table 7). However, the relative HA titre was more than 6.5 fold lower than expected.

Fluorescent antibody staining of FP/R infected erythrocytes

It was important to determine whether viral proteins were synthesized in all or only in a few particularly productive erythrocytes. This could be decided by immunofluorescence using antibody directed against the NP. In preliminary experiments levels of residual inoculum were sufficient to

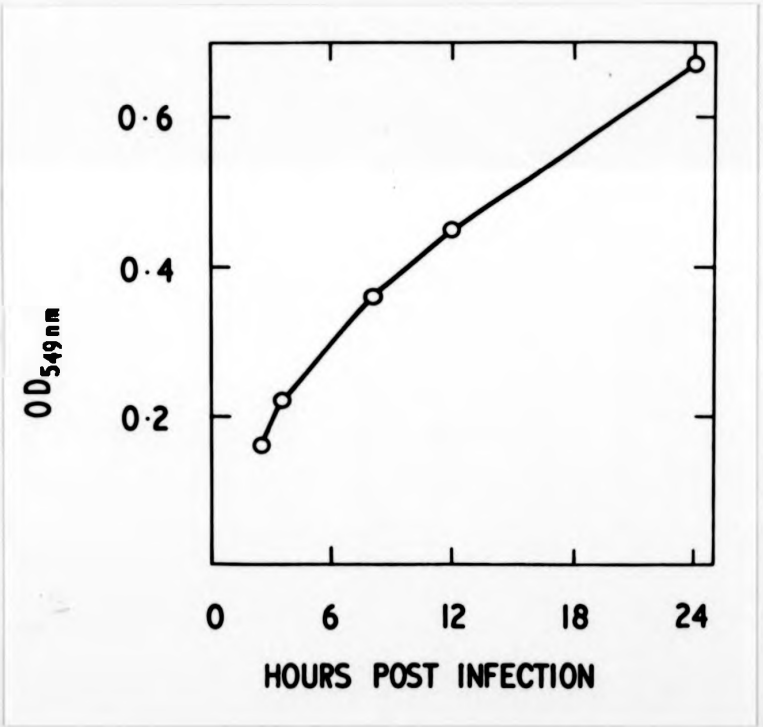

0·6
0·4
0·2
OD_{549nm}
0
6
12
18
24
HOURS POST INFECTION

Fig. 11

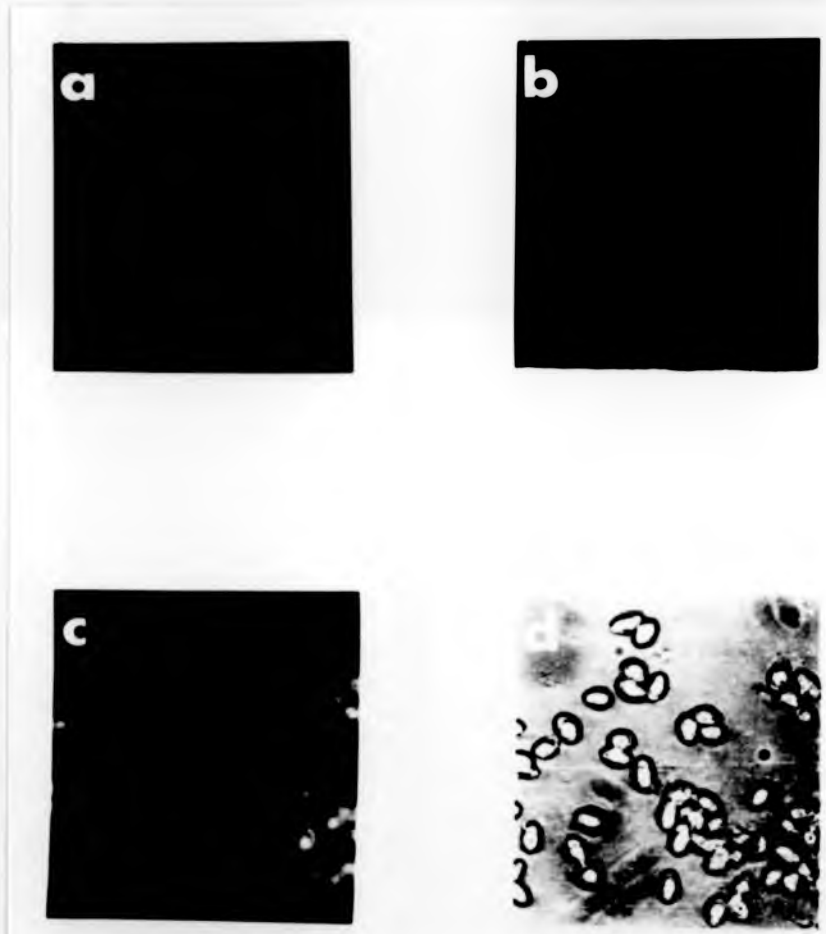
Synthesis of neuraminidase in FP/R infected erythrocytes

Samples of 10^7 erythrocytes taken at intervals after infection with FP/R were disrupted by sonication and assayed for the presence of NA by incubation with fetuin for 30 h at 37°C (Materials and Methods). The initial value probably represents residual inoculum.

cause fluorescence even at 1 h p.i. but this was reduced to near background levels with trypsin and an incubation at pH 3 (Materials and Methods). Although the level of fluorescence was low at all times compared with FP/R infected CEF cells there was a significant increase in fluorescence at 3 h p.i. (Fig. 12), which appeared to have increased further at 8 h p.i. Fig. 12 also shows that all erythrocytes fluoresced with the same intensity, indicating that infection and synthesis were uniform with respect to time and quantity of antigen produced. Furthermore, although fluorescence was too faint to be certain of its location it appeared at 8 h p.i. to be brightest in the region of the nucleus.

Synthesis of FP/R proteins in erythrocytes from embryo and adult chickens

Although histological examination of erythrocytes from 13 day old embryos (used above) identified the majority (> 80%) of them as mature erythrocytes there are indications that erythrocytes from older birds are less metabolically active (Romanoff, 1960). Therefore, purified erythrocytes from 20 day old and adult birds were infected to determine if they still possessed the cellular functions necessary for influenza virus protein synthesis. Fig. 13 shows that NP, M and NS1 could be resolved in erythrocytes from both these ages of chicken. However, radiolabelling showed that amounts of

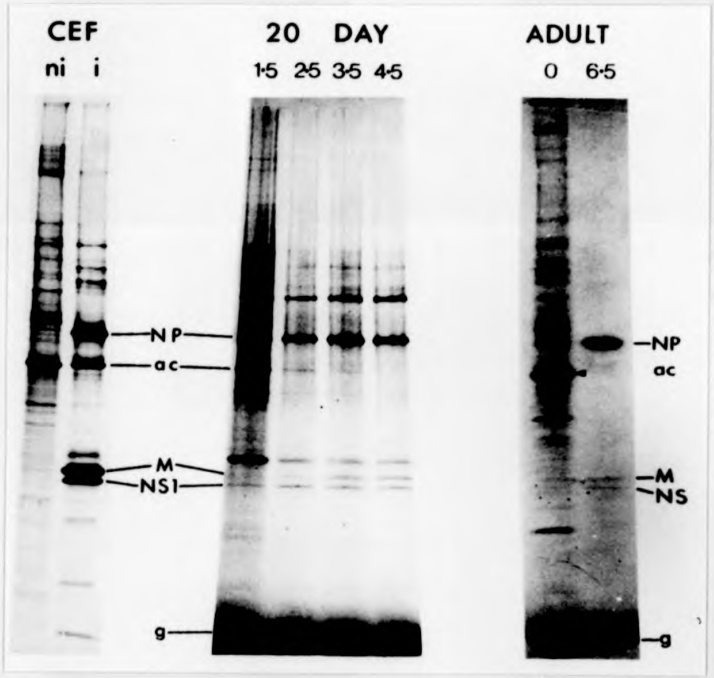

a
b
c

a
b
c

Fig. 12

Fluorescent antibody (anti NP) staining of FP/R infected erythrocytes

Fluorescent antibody staining of (a) non-infected erythrocytes, (b) infected erythrocytes at 3.5 h p.i. and (c) infected erythrocytes at 8 h p.i. with FP/R, (d) shows the same field as (c) viewed under phase contrast optics. All aspects of photography of stained cells were kept constant.


CEF
ni i
20 DAY
1·5 2·5 3·5 4·5
ADULT
0 6·5
NP
ac
M
NS1
g
NP
ac
M
NS
g

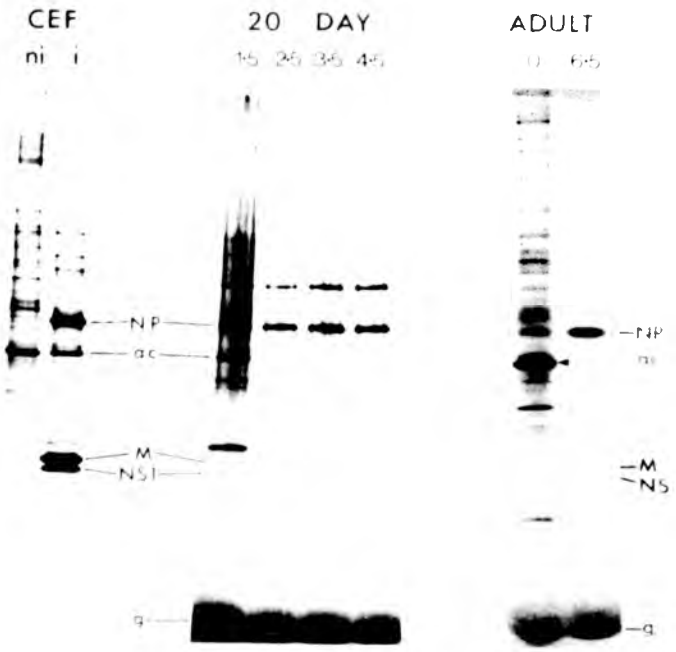

CEF
ni i
20 DAY
ADULT
NP
M
NS1
NP
M
NS

Fig. 13

PAGE of proteins synthesized in FP/R infected erythrocytes from 20 day old embryos and from an adult bird

Erythrocytes from 20 day old embryos were infected with FP/R (30 PFU/cell) and samples of 10^7 cells radiolabelled with ^{35}S-methionine (100 μCi/10^7 cells) for 30 min at 1, 2, 3 and 4 h p.i. FP/R infected (30 PFU/cell) erythrocytes from an adult bird were radiolabelled with ^{35}S-methionine (100 μCi/ml) 6 to 6.5 h p.i.

^{35}S-methionine incorporated into TCA precipitable material of erythrocytes from 20 day old embryos and adult birds was 30% and 10%, respectively, of that incorporated by erythrocytes of 13 day old embryos.

Multiplication of NDV and SFV in erythrocytes

Since no infectious FP/R virus was produced in infected avian erythrocytes, the question was raised as to whether these cells lacked some particular requirement for influenza replication or were incapable of forming enveloped progeny virus *per se*. This was tested by infecting avian erythrocytes with SFV and NDV. These viruses both have RNA genomes and NDV is a natural pathogen of fowl. After infection, erythrocytes were washed extensively and treated with trypsin as before. Residual NDV inoculum was further reduced by treatment with neutralizing antiserum for 1 h at 37°C and residual SFV by washing cells with pH 3 buffer solution. Samples of cells and culture fluids were disrupted at intervals after infection and infectivity measured by plaque assay on CEF monolayers. The experiment was continued until 24 h p.i. at which time in contrast to infection with FP/R practically all erythrocytes had lysed.

There was an increase in infectivity of about 100-fold of both NDV and SFV during the incubation time (Fig. 14) showing erythrocytes from 13 day old chicken embryos could sustain a productive infection by both viruses. However, as the maximum yield was 1 PFU/200 cells only a minority of cells in the erythrocyte population could be contributing to the yield of progeny virus.

Multiplication of NDV in erythrocytes from adult chickens

Perhaps of more interest from the disease point of view was whether NDV could productively infect the erythrocytes of the adult chicken. Blood was taken from the wing vein of adult birds and spun through 10% Ficoll to remove leucocytes. The experimental protocol for infecting with NDV, the reduction of inoculum virus and the disruption of samples for plaque assay on CEF monolayers was as for erythrocytes from 13 day old embryos.

As shown in Fig. 15 there was an increase in infectivity, similar to that observed with NDV infected embryo erythrocytes. Therefore, apparently some of the erythrocytes in the circulating blood of the adult bird are able to sustain a productive infection of NDV.

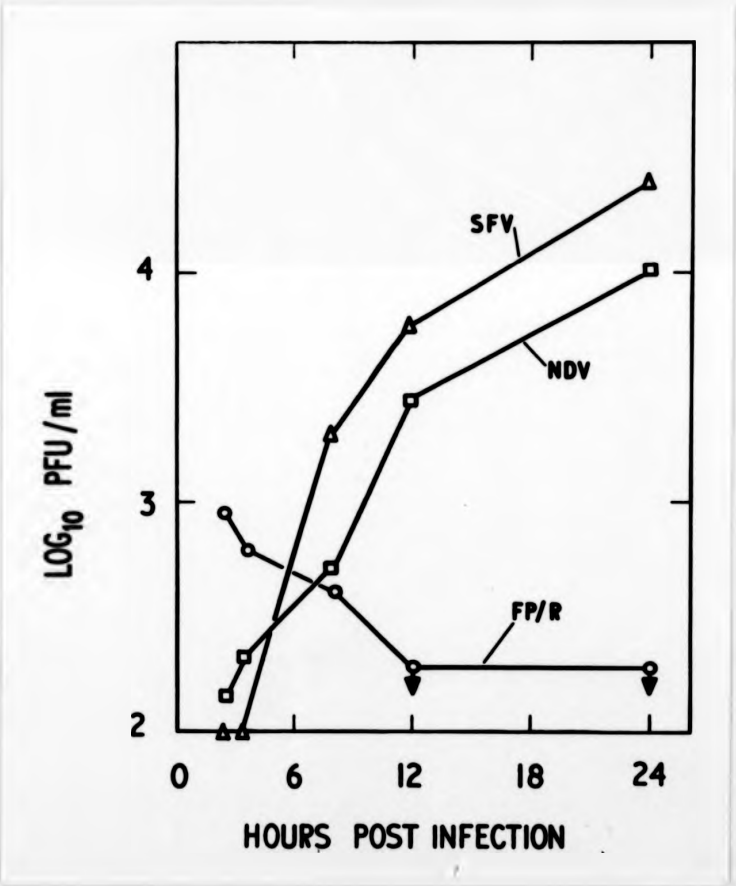

SFV
NDV
FP/R
4
3
2
0
6
12
18
24
LOG10 PFU/ml
HOURS POST INFECTION

Fig. 14

Multiplication of NDV, SFV and FP/R in erythrocytes from 13 day old embryos

Duplicate samples of 10^7 erythrocytes and their culture fluids were taken at intervals after infection with NDV-Texas, SFV or FP/R, disrupted by ultrasonication and assayed on CEF monolayers.

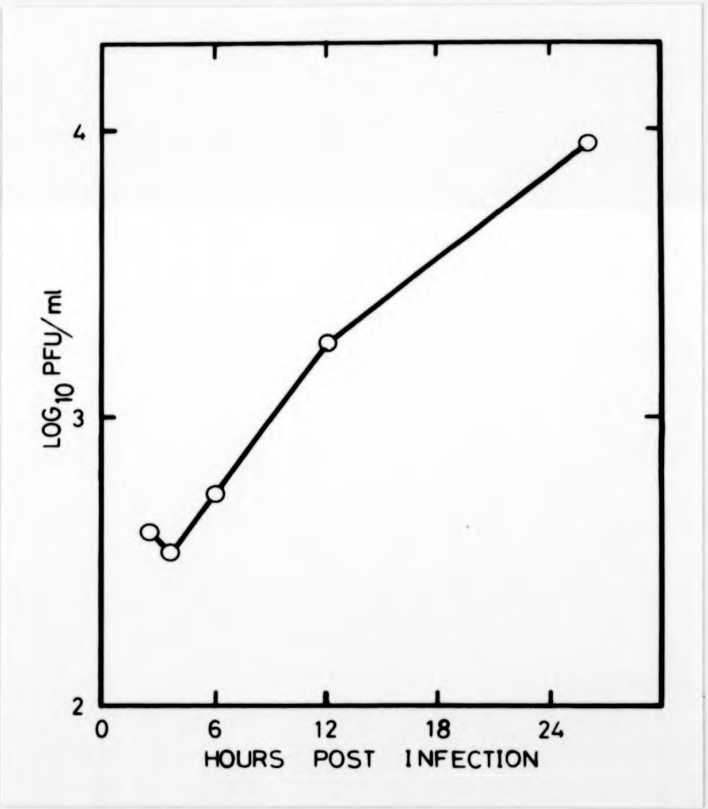

4
3
2
LOG10 PFU/ml
0
6
12
18
24
HOURS POST INFECTION

Fig. 15

Multiplication of NDV in erythrocytes from an adult chicken

Blood was removed from a wing vein and spun through 10% Ficoll to remove leucocytes. Erythrocytes (10^7 cells/ml) were infected at 30 PFU/cell and treated to remove inoculum virus as described for erythrocytes from 13 day old embryos.

Viral protein synthesized by 13 day old embryo erythrocytes infected with NDV and SFV

Since these viruses multiplied in erythrocytes we looked for the synthesis of viral protein by radiolabelling with ^{35}S-methionine and analysis by PAGE. NDV synthesized very little detectable viral protein (Fig. 16) and SFV none (compared with FP/R in Fig. 2 labelled under identical conditions) even at times when infectious virus production had increased by 100-fold. The major NDV polypeptide appeared in the 55-65,000 MW region as found in NDV-infected CHO cells (Clinkscales and Bratt, 1978).

Viral protein synthesized by 13 day old embryo erythrocytes infected with NDV and SFV

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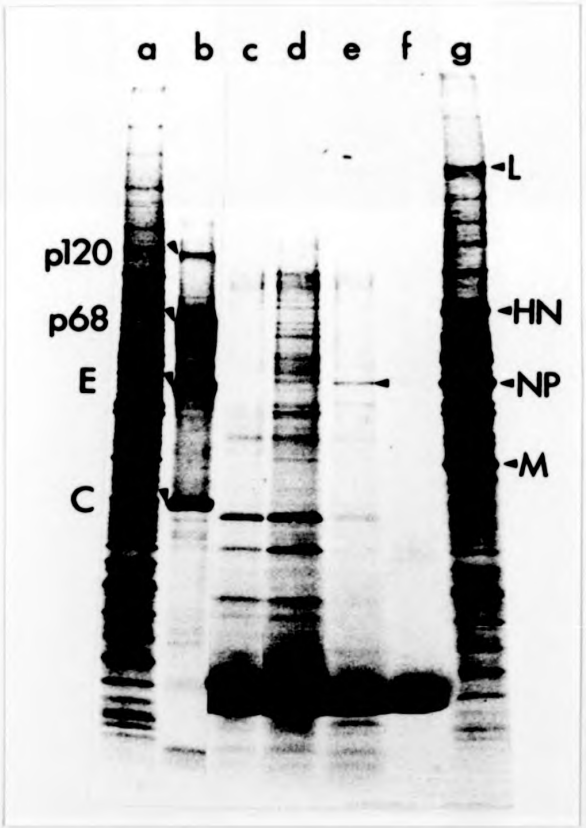

a b c d e f g
p120
p68
E
C
L
HN
NP
M

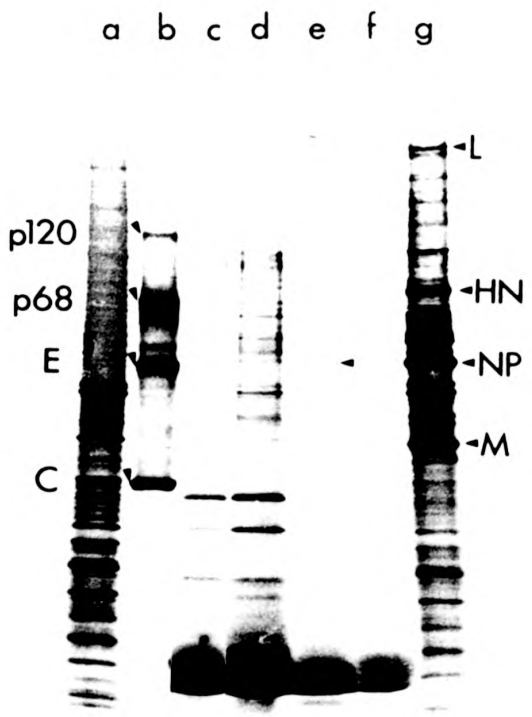

a b c d e f g
p120
p68
E
C
L
HN
NP
M

Fig. 16

Proteins synthesized by erythrocytes and CEF cells infected with NDV or SFV

PAGE of protein synthesized by erythrocytes and CEF cells infected with NDV or SFV under the same conditions used in Fig. 6 for FP/R. Viral proteins are arrowed.
a) Non-infected CEF cells; b), SFV-infected CEF cells; both a and b pulsed with ^{35}S-methionine from 6-6.5 h p.i.; c), SFV-infected erythrocytes pulsed from 9-9.5 h p.i.; d, non-infected erythrocytes; e, f, NDV-infected erythrocytes pulsed 3-3.5 and 9-9.5 h p.i. respectively; g, NDV-infected CEF cells. The experiments with c, e, f were done in the presence of 3 µg/ml actinomycin D to reduce endogenous protein synthesis and enhance the detection of viral proteins. Nomenclature of SFV and NDV protein follows the conventions of Clinkscales *et al*. (1978) and Bruton *et al*. (1976).

3. Discussion

Although there are numerous accounts of viruses becoming associated with erythrocytes (see General Introduction for references) either during or after infection this, to my knowledge, is the first account of de novo infection and synthesis of viral components within erythrocytes.

That erythrocytes and not leucocytes were responsible for FP/R protein synthesis was confirmed by obtaining a leucocyte preparation containing approximately 10^4 fold more leucocytes than unfractionated blood which failed to synthesize any detectable viral proteins.

It was, in fact, a surprise to see complete expression of FP/R proteins in erythrocytes, with P proteins, HA, NP, M and NS1 all synthesized in readily detectable amounts. Indeed, earlier work (Kelly and Dimmock, 1974 and Minor and Dimmock, 1976) suggested that the erythrocyte nucleus might not allow expression of HA and NA. However, this earlier work was carried out using erythrocytes fused with BHK cells which had been previously treated with AMD (Kelly and Dimmock, 1974) or enucleated by cytochalasin B (Minor and Dimmock, 1976). Therefore, it was possible that the fusion process or some other aspect of the experimental protocol caused inhibition of certain viral products or that the method of

detection, indirect fluorescent antibody staining, was not sensitive enough.

Evidence reviewed by Romanoff (1960) along with measurements on the incorporation of ^{35}S-methionine into TCA precipitable material (data not shown) suggested that erythrocytes from older embryos (20 days) and from adult birds were less metabolically active (but see Section 5). However, detection of NP, M and NS1 showed that even these erythrocytes allowed expression of the FP/R genome.

An alternative explanation for the data presented so far would be that FP/R proteins are only synthesized by a few particularly productive erythrocytes. For instance, the erythrocyte population of 13 day old chicken embryos contain approximately 20% immature cells (see Section 4) ranging from erythroblasts to almost fully differentiated late polychromatic erythrocytes. Therefore, it was considered likely that FP/R proteins were produced by immature rather than mature erythrocytes. This possibility was excluded, to a certain extent, by fluorescent antibody staining where NP antigen was detected at a uniform level in all erythrocytes.

Measurements based on incorporation of ^{3}H-leucine into TCA precipitable material indicated that FP/R infection (at least until 5 h p.i.) neither increases or decreases overall protein

synthesis in erythrocytes. This contrasts to some extent with FP/R infected CEF cells where there is a slight stimulation (1.5 fold) of protein synthesis (compared with uninfected cells) between 2 and 4 h p.i. (Skehel, 1972). However, in common with FP/R infected CEF cells (Borland and Mahy, 1968), RNA synthesis in erythrocytes was apparently stimulated (at least until 4 h p.i.) by FP/R infection. From this particular experiment it was not possible to distinguish whether this stimulation was due to viral or to host cell RNA synthesis.

Extraction of radiolabelled RNA and analysis by PAGE revealed that erythrocytes from 13 day old chicken embryos synthesize trace amounts (approximately 0.3% compared with an equivalent number of CEF cells) of 28S and 18S rRNA and in common with previous reports (Stephenson and Dimmock, 1974) the levels of these RNA species decline after infection with FP/R. Unfortunately, no viral RNAs could be detected in infected erythrocytes using this technique.

rRNA synthesis is reported to be absent in mature erythrocytes from adult birds (Longacre and Rutter, 1977). Detection of rRNA synthesis in erythrocytes from 13 day old embryos is therefore probably due to the presence of immature cells in the population (see Section 4) and/or to the possibility that those erythrocytes of 13 day old embryos classified as

morphologically mature may still retain some capacity for rRNA synthesis. This possibility was not investigated further.

As to the important question of whether infection of erythrocytes with FP/R resulted in the production of progeny virus it can be said that after reducing inoculum levels by 10^4 fold no increase in infectivity was detected. However, this still does not rule out production at very low levels.

The reason for the lack of detectable progeny virus did not emerge. However, the possibility that it was due to a failure of HA to be cleaved was eliminated by plaquing in the presence of trypsin which cleaves HA and activates infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Also the possibility that the treatment used to reduce the inoculum virus inhibited viral replication was countered by demonstrating the synthesis of FP/R proteins in surviving cells and that a similar treatment following infection with SFV did not prevent progeny virus formation.

A further possibility to explain absence of detectable progeny may be linked to the low level of functional HA detected (> 20-fold less than NP). The reason for this result is not clear since apparently normal amounts of HA protein were synthesized compared with other viral proteins (Fig. 6).

This being the case one would suspect that a post-translational event in the production of functional HA is affected. However, the receptor protein of NDV, which has to be both glycosylated and cleaved for infectivity (Homma and Ohuchi, 1973; Scheid and Choppin, 1974) is apparently produced normally.

Besides a block in HA production there are many other points in the multiplication cycle at which progeny virus production can be blocked. Evidence for this has been gathered from the study of different abortive or non-permissive infections where blocks in viral formation have been reported in vRNA replication (Avery, 1975); in transcription and in viral protein transport (Gandhi et al., 1971); in M protein synthesis (Valcavi et al., 1978) and in the final stages of maturation (Caliguiri and Holmes, 1979).

Although erythrocytes from 13 day old embryos and from adult birds produced progeny virus after infection with either NDV or SFV the titre was only 1 PFU/200 cells. This indicated that only a small proportion of the cells were productively infected. If this were so then it might explain why only low levels (compared with FP/R infection) of viral protein synthesis was detected, i.e. the proteins synthesized by the minority of infected cells being eclipsed by proteins synthesized by the majority of uninfected cells. In Section 4

an attempt is made to identify which cells are productively infected by separating erythrocytes according to their developmental status.

SECTION 2

The time course of synthesis of influenza proteins in chicken erythrocytes

1. Introduction

In the previous section it was established that avian erythrocytes from 13 day old embryo chickens would support the synthesis of fowl plague virus proteins. In this section the synthesis of these fowl plague proteins and the proteins of other influenza A viruses is investigated further.

2. Results

Erythrocytes from chicken embryos of the Legome Strain

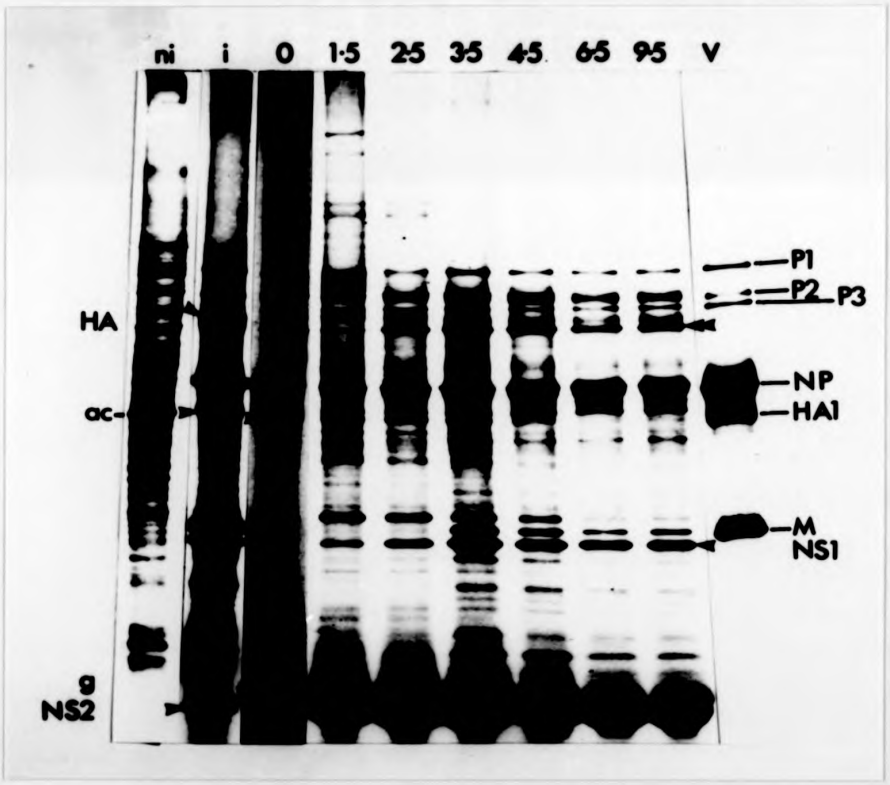
Soon after the experiments detailed in Section 1 had been completed, Locksley Ltd., ceased trading and a new supplier (Hawksley Chicks Ltd., Evesham) was found. The remaining experiments reported in this thesis are therefore with erythrocytes obtained from chickens and chicken embryos of Hawksley's Legome strain. Many of the experiments detailed in Section 1, such as investigations into the production of infectious NDV, SFV and FP/R plus the capacity to synthesize FP/R proteins, were repeated with erythrocytes from Legome chicken embryos. The results obtained (data not shown) were the same as for erythrocytes from the hybrid chicken strain of Locksley Ltd.

Time course of synthesis of FP/R proteins in pulse-labelled erythrocytes

Temporal control over viral protein synthesis has been observed in influenza virus infected cells (Skehel, 1972; Inglis et al., 1976). However, the time of appearance of influenza proteins varies depending on the virus strain and the cell type (Minor and Dimmock, 1979). To investigate

whether there was temporal control of protein synthesis in FP/R infected erythrocytes, cells were labelled with ^{35}S-methionine at intervals after infection (Fig. 17). P1, P2, P3, NP and NS1 were all detected after a 30 min pulse at 2.5 h p.i. and there was little change in amounts of P1-3 and NS1 synthesized up to 6.5 h p.i. M was first detected at 3.5 h p.i. showing that temporal control of protein synthesis exists in FP/R infected erythrocytes. A diffuse band co-migrating with uncleaved HA in FP/R infected CEF cells was first observed at 3.5 h p.i. Three protein bands (approximate MW 20,000, 15,000 and 14,500) could be seen migrating below NS1 in erythrocytes at 3.5 h p.i.. Equivalent protein bands were also detected in FP/R infected CEF cells (Cook et al., 1979). Proteins having similar MWs have been observed previously and all but one (NS2) shown to be cleavage products of NS1 (Lamb et al., 1978). Those in erythrocytes were not examined further.

In addition to a complete expression of the viral genome, FP/R infection of erythrocytes results in inhibition of cellular protein synthesis. This appeared to be more marked in the case of certain proteins than in others, e.g. four high MW (> 120,000) proteins at the top of the gel, a protein (MW 29,500) migrating just ahead of M protein and globin. However, a protein (arrowed ⟦◀◀⟧), presumably of cellular origin since it is present at time 0, appeared to increase


ni
i
0
1·5
2·5
3·5
4·5
6·5
9·5
V
P1
P2
P3
HA
NP
ac-
HA1
M
NS1
g
NS2

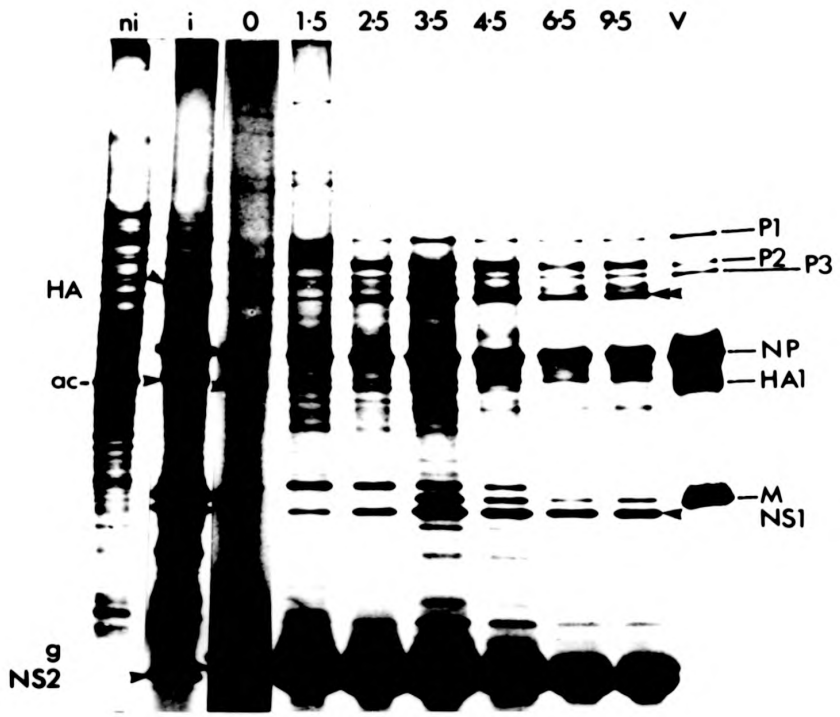

ni
i
0
1·5
2·5
3·5
4·5
6·5
9·5
V
HA
ac-
g
NS2
P1
P2
P3
NP
HA1
M
NS1

Fig. 17

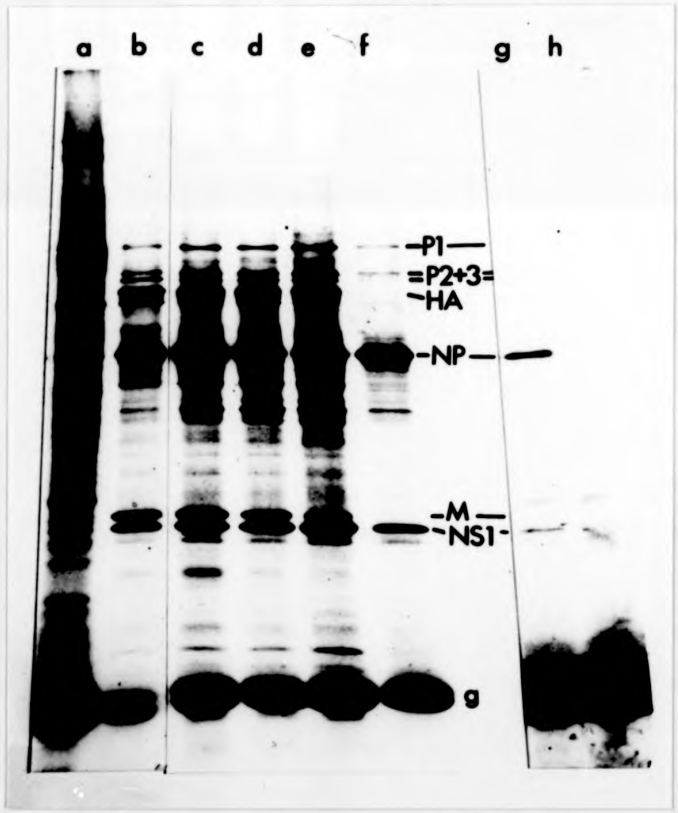
PAGE of proteins synthesized in FP/R-infected erythrocytes

Cells were infected with 30 PFU/cell and labelled with 100 μCi/ml/10^7 cells of ^{35}S-methionine for 30 mins. Erythrocytes were harvested at the times indicated in hours p.i. Infected (i) and non-infected (ni) CEF cells and ^{35}S-methionine-labelled virions (v) are included as markers.
ac = actin; g = globin.

after infection. This was not routinely observed and the reason for its apparent increase is not clear.

Because the erythrocyte nucleus is metabolically dormant compared to the nucleus of a CEF cell, it was a surprise to see such a rapid expression of the FP/R genome. However, it was possible that the nucleus was not participating since influenza virus gene expression occurs *in vitro* in coupled transcription-translation systems in the absence of nuclei (Content *et al.*, 1977; Minor and Dimmock, 1979). Against this possibility was the fact that in infected erythrocytes there is temporal control over the expression of FP/R proteins, a situation which does not occur in *in vitro* (Content *et al.*, 1977; Minor and Dimmock, 1979). However, it was decided to test that the erythrocyte nucleus was involved in the expression of the FP/R genome by pre-treating erythrocytes with AMD before infection. This drug has no effect on the *in vitro* coupled system (Minor and Dimmock, 1979), and although the mechanism whereby it inhibits influenza protein synthesis is not understood (see General Introduction) its site of action is within the host nucleus (Barry, 1964; Minor and Dimmock, 1975, 1977; Mark *et al.*, 1979; Barrett *et al.*, 1979).

Concentrations of AMD of 1 μg/ml and above abolished the synthesis of virus proteins in erythrocytes (Fig. 18). Lower


a
b
c
d
e
f
g
h
P1
P2+3
HA
NP
M
NS1
g

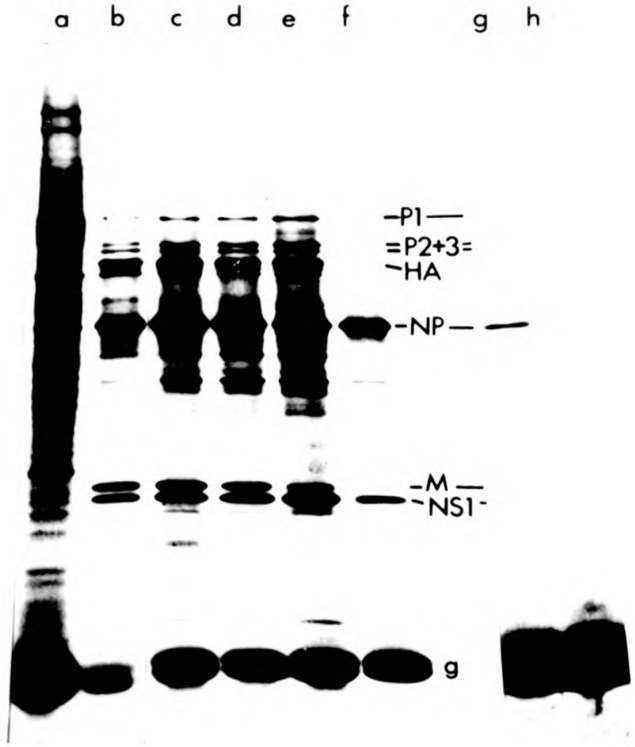

a b c d e f g h
P1
P2+3
HA
NP
M
NS1
g

Fig. 18

Effect of AMD on the synthesis of FP/R proteins in erythrocytes

Inhibition of synthesis of FP/R proteins in erythrocytes pretreated with various concentrations of AMD for 1 h before and throughout infection. Cells were labelled from 5 to 6 h p.i. with ^{35}S-methionine (100 μCi/10^7 cells).

(a) non-infected, (b) infected cells without AMD , with AMD at 0.01 (c), 0.03 (d), 0.1 (e), 0.3 (f), 1.0 (g) and 3.0 μg/ml (h). a to f and g, h are from separate experiments.

concentrations of the drug preferentially inhibited the synthesis of M and HA. This pattern of inhibition has been observed previously in BHK cells (Minor and Dimmock, 1975). Therefore it is concluded that the erythrocyte nucleus participates in the expression of the FP/R genome.

With the finding that erythrocytes would support FP/R protein synthesis we investigated the expression of the genomes of non-avian influenza strains.

Infection of avian erythrocytes with human influenza viruses

Figure 19a shows the pattern of protein synthesis in erythrocytes infected with A/WSN (H0 N1). Although NP and NS1 were present from 3.5 h p.i., M could not be detected even at 9.5 h p.i. A similar situation was observed with the H2 N2 strain A/Japan/305/57 (A/Jap) (Fig. 19b). This contrasts with the time course of FP/R protein synthesis where M was detected at 3.5 h p.i. (Fig. 17). Infection of erythrocytes with the H3 N2 influenza strain A/Hong Kong/1/68 (A/HK) revealed a different situation (Fig. 19c). Here the protein which co-migrated with NS1 from infected CEF cells was present in greater amounts than the protein co-migrating with NP. Furthermore, it would appear from Figs. 19a, b and c that erythrocyte protein synthesis overall, was not

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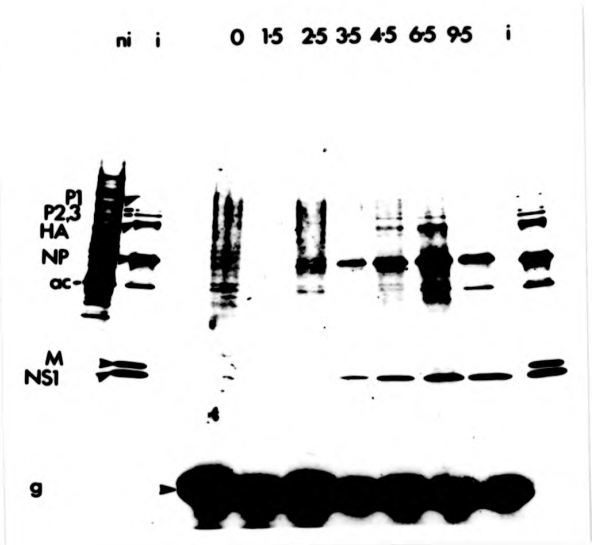
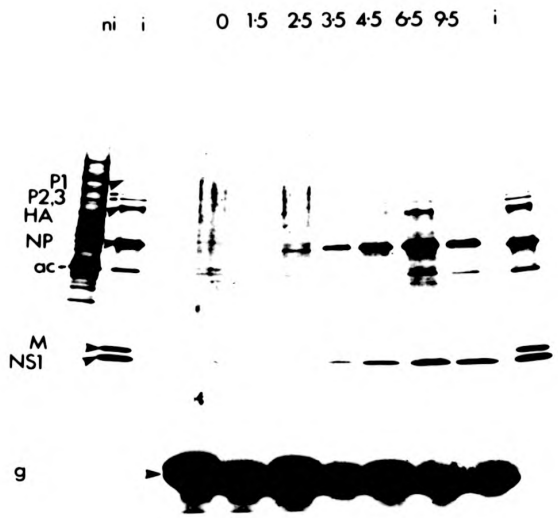
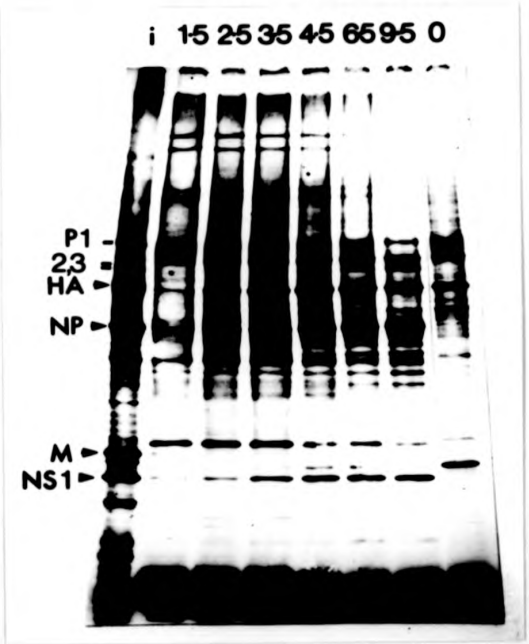
A



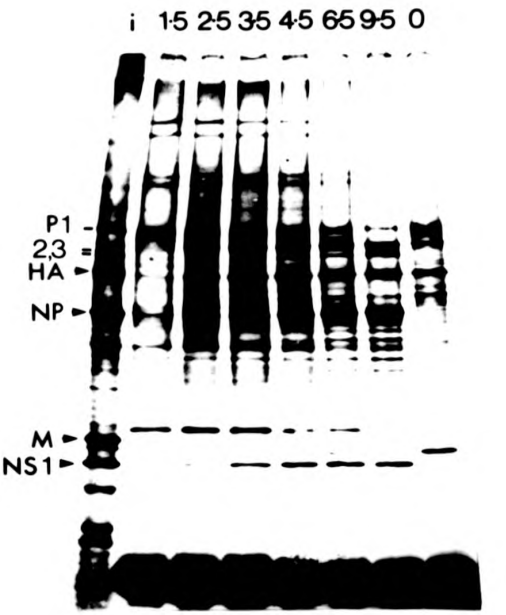
A



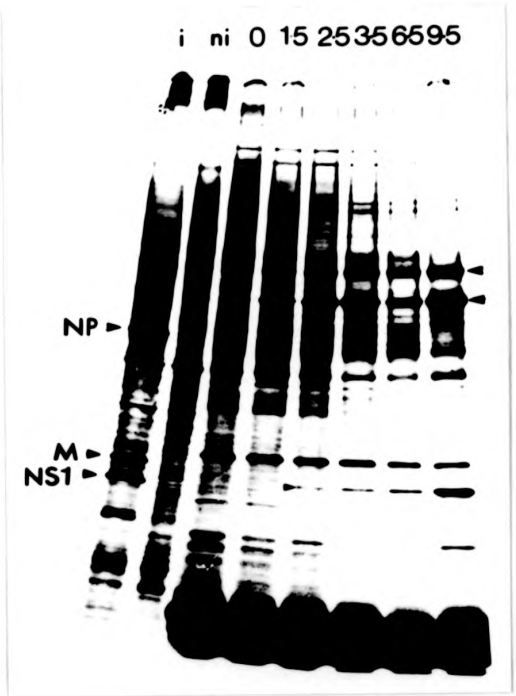
B



B



C



C

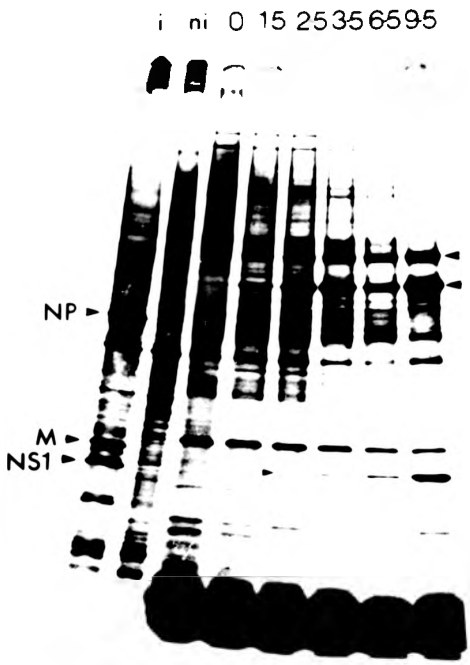


Fig. 19

Synthesis of proteins by human strains of type A influenza in erythrocytes of 13 day old embryos

Erythrocytes were infected and radiolabelled as described in Fig. 17. Infected (i) and non-infected (ni) CEF cells are included as markers.

A. A/WSN (moi = 30 PFU/cell)

B. A/Jap (moi = 300 HAU/10^7cells)

C. A/HK (moi = 300 HAU/10^7 cells)

significantly inhibited as a result of infection with human influenza strains. In fact, infection with A/Hong Kong seemed to have stimulated the synthesis of certain erythrocyte proteins (arrowed in Fig. 19c).

Mixed infection of erythrocytes by FP/Dutch and A/NWS: complementation for the synthesis of A/NWS M protein

The data just presented (Fig. 17 and Fig. 19) show that M protein is expressed in erythrocytes when infected with FP/R but not when infected with human influenza strains. This finding suggested an experiment to see if infection of erythrocytes with a fowl plague strain at the same time as infection with a human strain could evoke expression of the human strain M protein. Initially such an experiment proved difficult because M proteins from many influenza A virus strains were found to have similar mobilities on PAGE (data not shown). However, the avian influenza strain FP/Dutch and the human strain A/NWS were discovered to have M proteins that differed in electrophoretic mobility. Fig. 20A shows that FP/Dutch synthesized M protein in erythrocytes and that A/NWS did not. In the mixed infection (Figs 20B and C) the M proteins of both FP/Dutch and A/NWS were expressed.

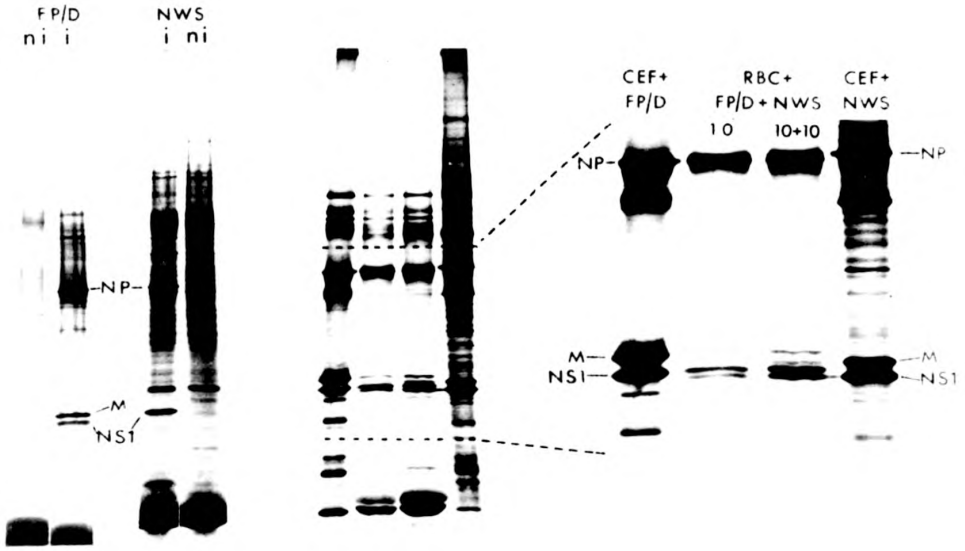

FP/D
ni i
NWS
i ni
NP
M
NS1
CEF+
FP/D
RBC+
FP/D + NWS
1 0
10+10
CEF+
NWS
NP
M
NS1
NP
M
NS1

Fig. 20

Synthesis of M proteins in erythrocytes following mixed infection with the avian strain FP/Dutch and the human strain A/NWS

A: Erythrocytes infected (i) or non-infected (ni) with FP/Dutch (20 PFU/cell), or A/NWS (300 HAU/10^7 cells) and labelled from 4 to 4.5 h p.i.

B: Mixed infection experiment of which part of the gel is photographically enlarged (C). Outer tracks: infected CEF markers; inner tracks: mixed infection pulsed for 10 min or pulsed and chased for an additional 10 min (10 + 10) at 4 h p.i.

3. Discussion

Both the kinetics of synthesis of FP/R proteins and the shut-off of cell proteins in erythrocytes were similar to those found earlier in CEF cells (Minor et al., 1979) indicating that in this regard virus expression was unaffected by the unusual structural and metabolic features of erythrocytes. Electron micrographs revealed (Wignall and Dimmock, unpublished data) that, in common with erythrocytes from adult birds (Fawcett and Witebskey, 1964; Schjeide et al., 1964; Zentgraf et al., 1971 and Blanchet, 1974) erythrocytes from 13 day old embryos lacked endoplasmic reticulum, possessed few mitochondria and had nuclei containing condensed chromatin. Furthermore, these embryo derived erythrocytes synthesized little DNA and RNA compared to CEF cells (see Section 4). That the erythrocyte nucleus was involved in FP/R gene expression, as it is in relatively non-differentiated and dividing cells (Follett et al., 1974; Kelly et al., 1974), was shown by the inhibition of viral protein synthesis caused by pre-treating erythrocytes before infection with AMD.

In contrast to the situation with FP/R, the human influenza strains tested failed to synthesize M protein in erythrocytes. The inability of certain cell lines to synthesize M protein has been reported by others (Bosch et al., 1978; Lohmeyer et al., 1979; Valcavi et al., 1978) but this is the first

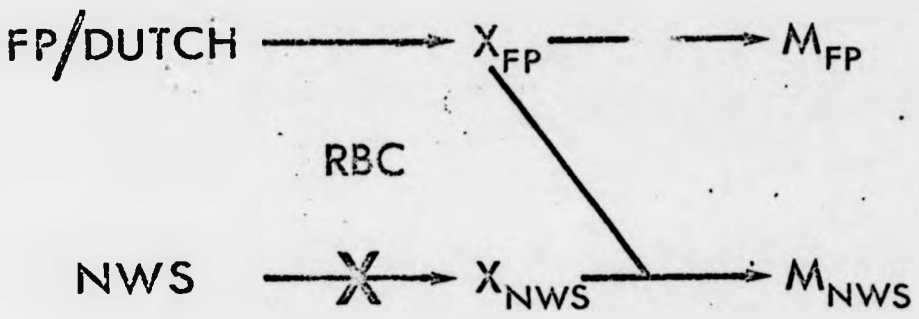
instance of the defect being virus strain specific. Furthermore, A/HK/1/68 (H3 N2) synthesized NS1 in greater amounts than NP. These data underline the point of Minor *et al*. (1979) that expression of viral proteins is dependent on both host cell and the virus strain.

It was not determined whether synthesis of human influenza M was blocked at the level of transcription or translation. Unfortunately, viral mRNAs extracted from infected erythrocytes could not be sufficiently radiolabelled to be analysed by PAGE (see Section 1). However, there is much evidence to suggest that control of influenza protein synthesis is at the level of transcription (Glass *et al*., 1975; Hay *et al*., 1977; Taylor *et al*., 1977; Inglis and Mahy, 1979) and so it is probable that there is a block during secondary or amplified transcription (see General Introduction) in the production of messenger cRNA for human influenza M protein. An experimental protocol which may be of use in testing this hypothesis would be to prepare vRNA encoding M protein by electrophoresis, to radiolabel it and use it as a probe for the detection of M protein message. That this approach may have the necessary sensitivity has been shown by its success in the study of primary transcription (Mark *et al*., 1979; Inglis and Mahy, 1979).

Since FP/R synthesized M while human influenza strains did not a 'complementation' experiment was set up in which erythrocytes were infected with both a human and an avian influenza strain simultaneously. In such a system the M proteins of both human and avian strains were synthesized in comparable amounts (Fig. 20). Thus, it appears in the synthesis of M protein there is an interaction (as yet undetermined) between a host specified factor(s) and a virally specified factor(s). The existence of a viral factor(s) is postulated since M protein synthesis is virus strain specific and the existence of a host factor(s) because although human influenza M is not synthesized in erythrocytes it is synthesized in CEF cells. On this basis a model can be proposed to explain the 'complementation' between avian and human influenza strains (see Fig. 21).

Since human influenza strains might be considered to have evolved to replicate in human cells, it is envisaged that the host-virus interaction(s) leading to M protein synthesis occur less effeciently in the human strain/avian cell system than in the avian strain/avian cell system. With infection of CEF cells by human influenza strains the less efficient interaction is assumed not to matter since it is compensated by there being an 'excess' of host factor(s). However, as erythrocytes mature it is envisaged that the amounts of the necessary host factor(s) are reduced such that the inefficiency

Fig. 21



X = VIRAL PRODUCT REQUIRED FOR SYNTHESIS OF M

Model explaining how co-infection of erythrocytes with NWS and FP/Dutch may result in expression of the NWS M protein

of the host factor/virus factor interaction is not compensated for and so consequently M protein is not synthesized. With avian influenza strains the efficiency of interaction between avian host and virus factors is such that the effects of erythrocyte maturation have little effect on M protein synthesis.

An alternative model based on the concept of host/virus factor interaction is that human influenza strains utilize a different host factor from that of avian strains. Therefore, both types of host factor would be present in CEF cells but only the avian type in erythrocytes.

These models assume either a reduction or a lack of the necessary host factor in erythrocytes as a result of differentiation. This could be tested by separation of the cells of the erythropoietic series and determining if cells early in the series allow M protein synthesis when infected with a human influenza strain (see Section 4).

It would of course be of considerable interest to identify the host and viral factors involved. An approach to identify the viral factor would be to generate a series of recombinant virus strains in which each gene of a human strain was substituted in turn by the equivalent gene from an avian strain. Thus, if only a single viral gene was involved, it

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would be possible to see which of the avian influenza genes resulted in M synthesis in erythrocytes. If such an approach was successful and the viral factor identified it may then be possible to use it as a 'probe' for the host factor(s) involved.

SECTION 3

The distribution of viral proteins in the nuclei of FP/R infected chicken embryo cells

1. Introduction

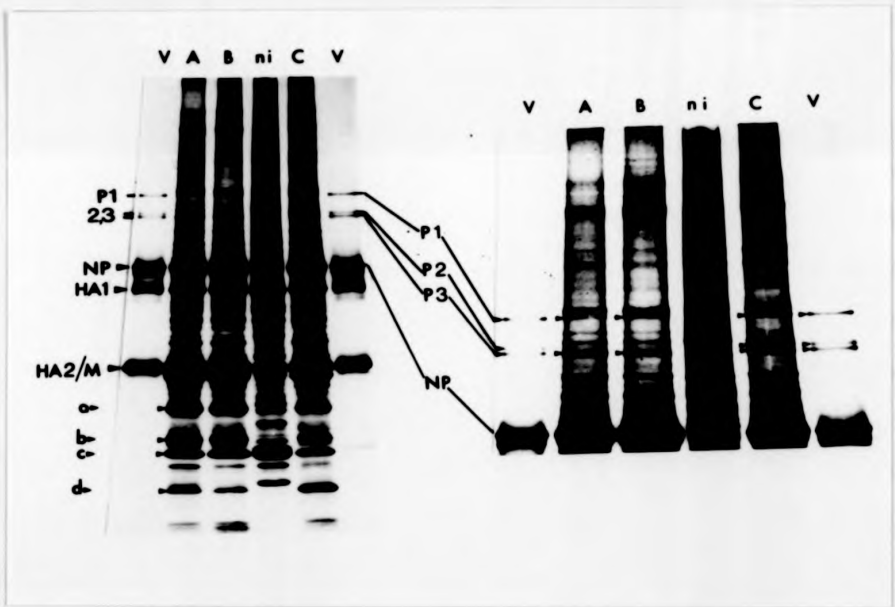
Viral proteins have been shown to migrate to the cell nucleus during the course of influenza infection (Breitenfeld and Schafer, 1975; Dimmock, 1969; Maeno and Kilbourne, 1970; Oxford and Schild, 1975). In view of this the distribution of proteins in the nuclei of influenza-infected erythrocytes was investigated to see if the quiescent nature of the erythrocyte nucleus created any major differences from proteins found in the nuclei of other infected cell types. In cell types already examined NP and NS1 are always observed in the nucleus, while HA and NA remain cytoplasmic (Breitenfeld and Schafer, 1957; Dimmock, 1969; Maeno and Kilbourne, 1970; Oxford and Schild, 1975). The situation with M protein is less clear. M has been reported to be almost entirely cytoplasmic in MDCK cells infected with WSN (Krug and Etkind, 1973; Krug and Soeiro, 1975) or fowl plague virus (Mahy et al., 1980) but to accumulate in the nucleus of WSN or fowl plague virus-infected chick embryo cells (Gregoriades, 1973; Hay and Skehel, 1975; Flawith, 1979; Flawith and Dimmock, 1979) while in another system strain X31 in MDBK cells, M antigen was detected in the cytoplasm except in a few cells where a proportion of the antigen appeared in the nucleus (Oxford and Schild, 1975).

Also subject to uncertainty is the intracellular location of the P proteins. P proteins were found in the nuclei of WSN infected MDCK cells (Krug and Etkind, 1973) but only in trace amounts in the nuclei of FPV infected chick embryo cells (Hay and Skehel, 1975). A possible explanation for this may lie in differences between virus strains and cell types. For example, the time after infection at which observations were made were similar for both groups. Krug and Etkind (1973) looked at nuclei 4 to 6 h p.i. whereas Hay and Skehel (1975) looked at times from 5 to 6 h p.i. Furthermore, both groups used Dounce homogenization to fractionate nuclei. However, the data presented by Krug and Etkind (1973) and Hay and Skehel (1975) suffered from certain technical difficulties. P proteins are synthesized in relatively low amounts compared to other viral proteins making detection difficult. Secondly, the polyacrylamide gel system used by both groups to detect P proteins did not resolve them into three distinct bands. Hay and Skehel (1975) could detect two bands whereas Krug and Etkind (1973) could detect only one. In view of these difficulties the distribution of P proteins found in FP/R infected CEF cells and erythrocytes was investigated.

2. Results

Distribution of viral P proteins in nuclei of FP/R infected CEF cells

Figure 22 shows the overall distribution of viral proteins in nuclei at various times after labelling with ^{35}S-methionine. Nuclei isolated immediately after the pulse contained NP, M and NS1 as previously reported (Flawith and Dimmock, 1979). Also present in nuclei were at least four proteins migrating ahead of NS1 (labelled a-c) which were similar in size to those reported by others (Skehel, 1972; Lamb and Choppin, 1978). A photographic enlargement of the region of the gel resolving the high MW proteins was made to more easily distinguish between viral and cellular proteins. After pulsing at 4 h p.i. for 10 min P1, P2 and P3 were present in approximately equal amounts in whole cell extracts but isolated nuclei contained only P1 and P3. Thus at this stage P_2 was confined to the cytoplasm. There was no change in distribution of the P proteins after an additional 10 min incubation in unlabelled medium, but after 2 h incubation (to 6 h p.i.) P_2 was present in nuclei in about the same amounts as P1 and P3. Thus as regards transfer to the cell nucleus there is a difference in the behaviour of P_2 from P1 and P3.


V A B ni C V
P1
2,3
NP
HA1
HA2/M
V A B ni C V
P1
P2
P3
NP

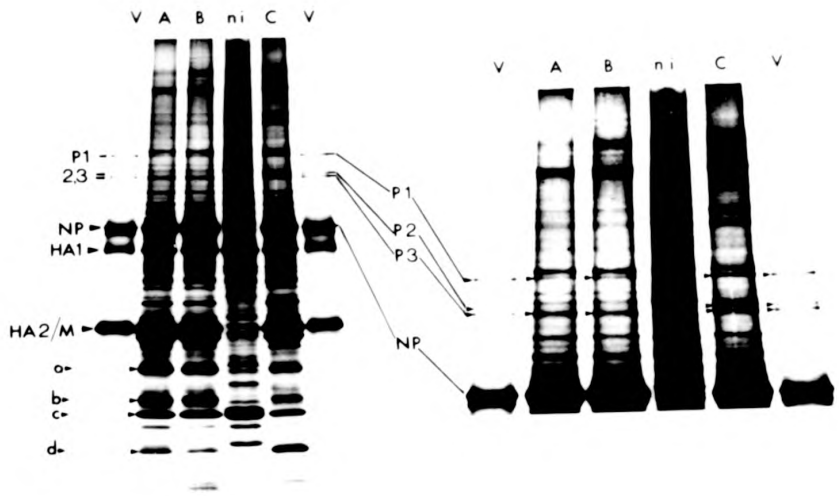

V A B ni C V
P1
2,3
NP
HA1
HA2/M
a
b
c
d
V A B ni C V
P1
P2
P3
NP

Fig. 22

PAGE of proteins from nuclei of CEF cells infected with FP/R

Infected cells were radiolabelled for 10 min with ^{35}S-methionine at 4 h p.i. (A); incubated for an additional 10 min in the absence of radiolabel (B); and for an additional 2 h in the absence of radiolabel (C). Non-infected cells (ni) were labelled for 10 min in parallel with (A). Marker radiolabelled virions (V) were also analysed.

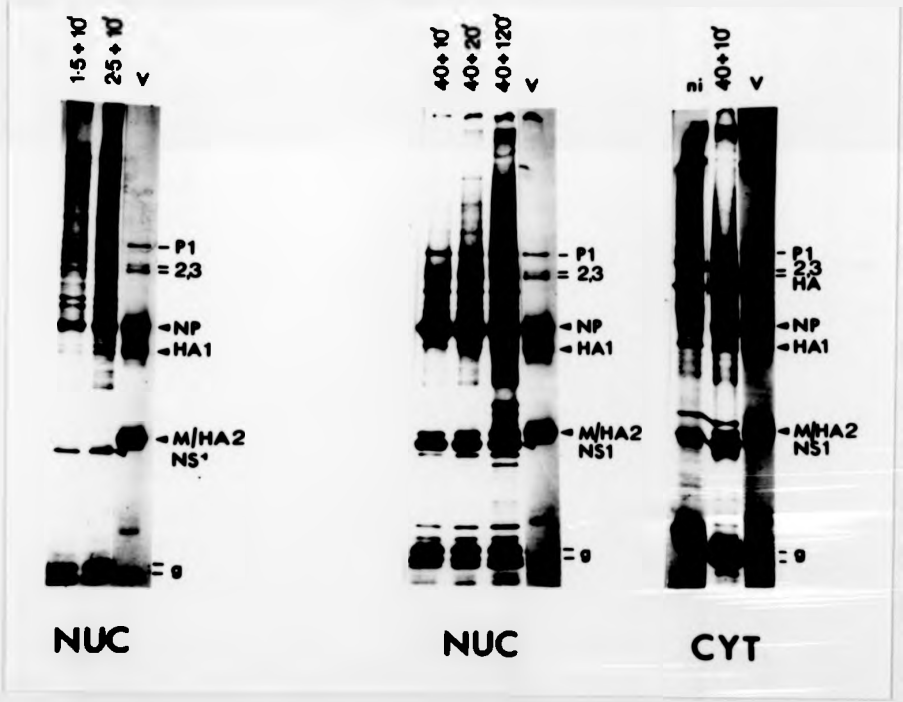
The right hand panel shows a photographic enlargement of the region containing the P proteins.

A different situation has been reported in FP/R infected MDCK cells in that less P3 appears to migrate to the nucleus than P1 or P2 (Mahy et al., 1980). Thus, migration of P proteins may well show variation depending on the cell line.

Viral proteins in nuclei of FP/R infected erythrocytes

Nuclei isolated from FP/R infected erythrocytes, which were radiolabelled at 1.5 or 2.5 h p.i., contained detectable amounts of NP and NS1 (Fig. 23). At these times P proteins could not be detected against the background of host proteins. However, nuclei isolated after radiolabelling at 4 h p.i. contained P proteins, NP, M and NS1, but not HA. HA was being synthesized at this time as evidenced by its presence in the cytoplasm (Fig. 23). The 4 h nuclear fractions also show that some globin was present in the nucleus. This does not necessarily denote cytoplasmic contamination as globin has been observed in the nucleus by others (Davies, 1961; Small and Davies, 1972).

If incubation of erythrocytes radiolabelled at 4 h p.i. was continued in ^{35}S-methionine free medium for periods of upto 2 h (i.e. to 6 h p.i.) it appeared that amounts of FP/R


1·5 + 10'
2·5 + 10'
v
P1
= 2,3
NP
HA1
M/HA2
NS1
= 0
NUC
40 + 10'
40 + 20'
40 + 120'
v
P1
= 2,3
NP
HA1
M/HA2
NS1
= 0
NUC
ni
40 + 10'
v
P1
= 2,3
HA
NP
HA1
M/HA2
NS1
= 0
CYT

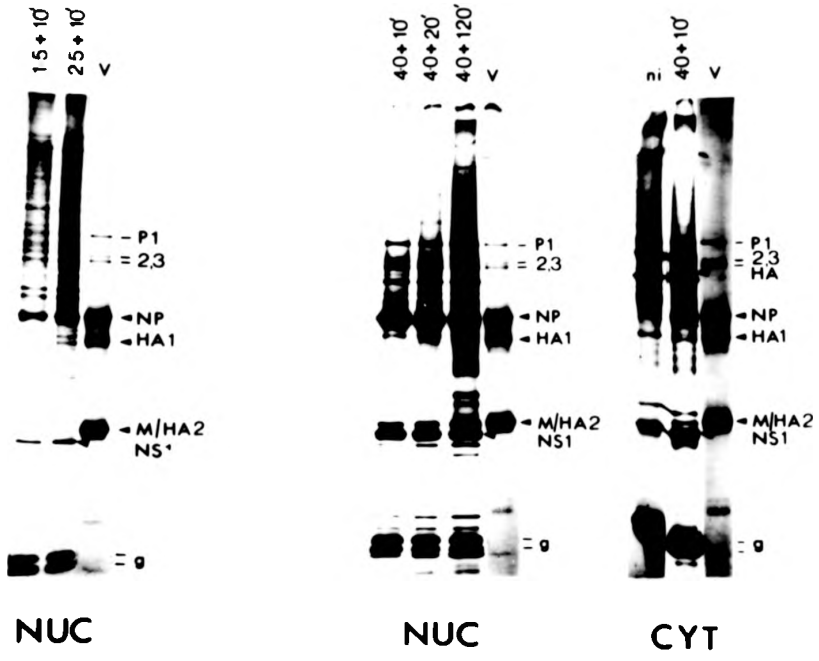

1.5+10'
2.5+10'
V
P1
2,3
NP
HA1
M/HA2
NS1
g
NUC
40+10'
40+20'
40+120'
V
P1
2,3
NP
HA1
M/HA2
NS1
g
NUC
ni
40+10'
V
P1
2,3
HA
NP
HA1
M/HA2
NS1
g
CYT

Fig. 23

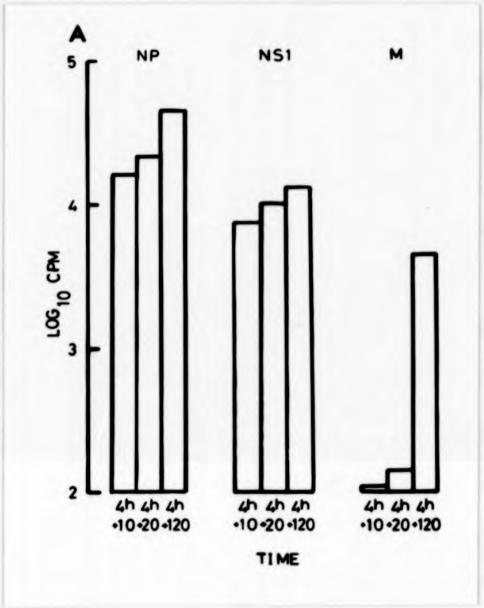
PAGE of proteins from nuclei and cytoplasms of FP/R infected erythrocytes

Cells were infected and radiolabelled for 10 min with ^{35}S-methionine (100 μCi/10^7 cells) at 1.5 h p.i., (1.5 + 10'), 2.5 h p.i. (2.5 + 10') and at 4 h p.i. (4 + 10'). Infected erythrocytes radiolabelled at 4 h p.i. were incubated for an additional 10 min (4 + 20') and 120 min (4 + 120') in the absence of radiolabel. Cytoplasmic fractions were from uninfected erythrocytes and from erythrocytes radiolabelled at 4 h p.i. (4 + 10'). Radiolabelled virions were included as markers.

Although all samples were prepared during the same experiment tracks labelled 1.5 + 10' and 2.5 + 10' were from a different gel than 4 + 10', 4 + 20' and 4 + 120'.

proteins in the nucleus, with the possible exception of M, remained relatively constant. This was checked by measuring the radioactivity in the areas of the gel corresponding to the protein bands NP, M and NS1 (Materials and Methods). Fig. 24a shows amounts of NP, M and NS1 present in nuclear fractions while Fig. 24b shows amounts of the proteins present in the equivalent cytoplasmic fractions, figures were normalized as detailed in Materials and Methods. The total amounts of radioactivity in the protein bands increased in both nuclear and cytoplasmic fractions with time, showing that this was not a true pulse-chase experiment. This was particularly evident for M protein where substantial increases in amounts (approximately 12-fold) were seen after the 2 h additional incubation.

Directly after the labelling period and after an additional 10 min incubation in ^{35}S-methionine-free medium the amounts of NP, M and NS1 were very similar in both nuclear and cytoplasmic fractions. After an additional 2 h incubation, amounts of NP and M were 2-fold higher and amounts of NS1 were 5-fold higher in the nuclear fractions. With the possible exception of measurements taken after the 2 h additional incubation it is striking how closely nuclear and cytoplasmic levels of NP, M and NS1 parallel each other. This was not observed in FP/BEL infected CEF cells where after the period of radiolabelling nuclear and cytoplasmic levels of NP, M and NS1 showed distinct differences (Flawith, 1979; Flawith and Dimmock, 1979).


A
NP
NS1
M
5
4
3
2
LOG10 CPM
4h 4h 4h
•10 •20 •120
TIME

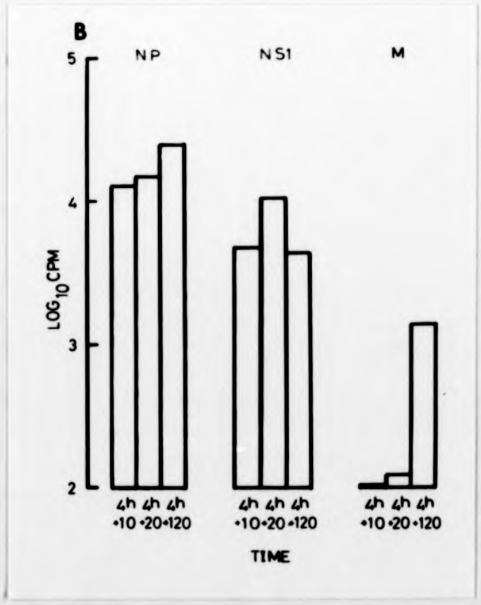

B
NP
NS1
M
5
4
3
2
LOG10 CPM
4h 4h 4h
•10 •20 •120
TIME

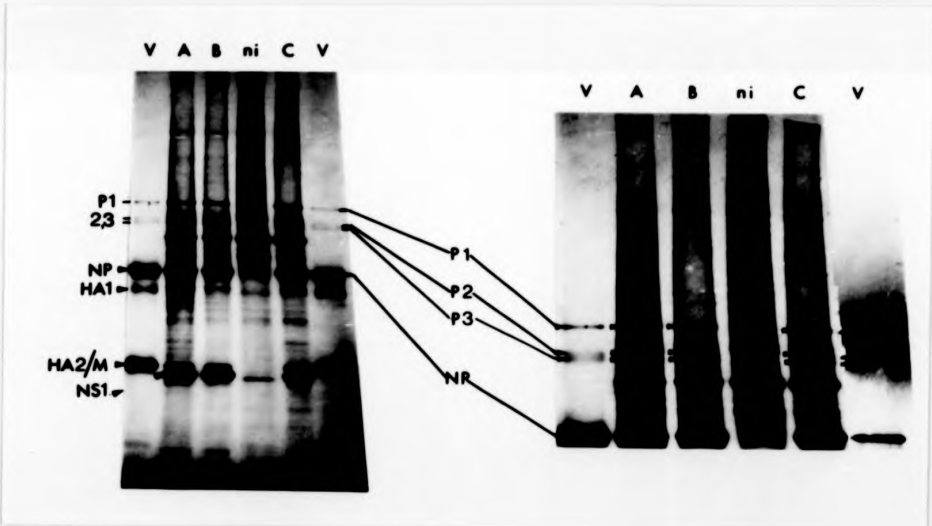
Fig. 24

Relative amounts of NP, NS1 and M in the nucleus and cytoplasm of FP/R infected avian erythrocytes

A. Shows amounts of NP, NS1 and M contained in the nuclear fractions of erythrocytes (shown in Fig. 23) which were radiolabelled at 4 h p.i. and incubated for either 10, 20 or 120 minutes.

B. Shows amounts of NP, NS1 and M from the equivalent cytoplasmic fractions.

Figure 23 shows that P1,2,3 are present in the nucleus directly after radiolabelling at 4 h p.i.. This was checked by preparing fresh samples of FP/R infected erythrocyte nuclei, radiolabelled at 4 h p.i. (Fig. 25). A photographic enlargement of that region of the gel resolving high MW proteins is included to make it easier to see the viral proteins. P1, P2 and P3 were all present in infected erythrocytes directly after radiolabelling and were there at 6 h p.i. Thus, unlike CEF cells, P1,2,3 migrate to the cell nucleus at the same rate.


V A B ni C V
P1
2,3
NP
HA1
HA2/M
NS1
V A B ni C V
P1
P2
P3
NR

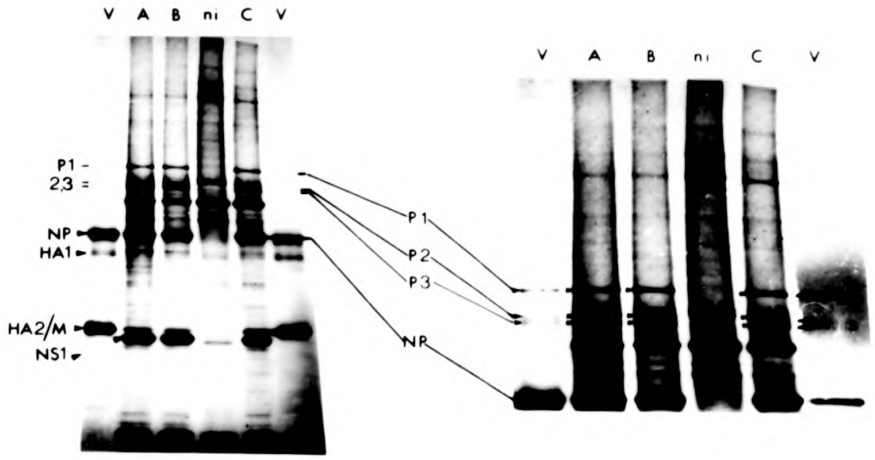

V A B ni C V
P1 -
2,3 =
NP
HA1
HA2/M
NS1
P1
P2
P3
NP
V A B ni C V

Fig. 25

PAGE of proteins from nuclei of erythrocytes infected with FP/R

Infected cells were radiolabelled for 10 min with ^{35}S-methionine at 4 h p.i. (A); incubated for an additional 10 min in the absence of radiolabel (B); and for an additional 2 h in the absence of radiolabel (C). Non-infected cells (ni) were labelled for 10 min in parallel with (A). Radiolabelled virions (V) were analysed as markers.

The right hand panel shows an enlargement of the region containing the P proteins.

3. Discussion

In FP/R infection of CEF cells P2 behaves differently from P1 and P3 and is transferred at a slower rate to the cell nucleus (Fig. 22). All three P proteins have been implicated in the synthesis of vRNA (reviewed by Scholtissek, 1978; Barry and Mahy, 1979) and so their migration to the nucleus is consistant with the suggestion that vRNA synthesis occurs there (Mahy et al., 1975; Assadullaef et al., 1975; Armstrong and Barry, 1974; Avery, 1974). However, not enough is known about the synthesis of vRNA to determine whether the differential migration of P2 has any functional significance in its production in CEF cells. Furthermore, since mutations in the gene coding for P2 prevent synthesis of all types (vRNA, Poly A cRNA and Non-Poly A cRNA) of influenza RNA within infected cells (reviewed by Scholtissek, 1978) it is possible that the behaviour of P2 reflects some other, as yet unidentified, role.

In FP/R infected erythrocytes the P proteins, NP, M and NS1 but apparently not HA, were found in the nucleus. This agrees with previous reports on the nuclear association of influenza proteins (Breitenfeld and Schafer, 1957; Dimmock, 1969; Maeno and Kilbourne, 1970; Gregoriades, 1973; Hay and Skehel, 1975). In FP/BEL infected CEF cells clear differences could be seen in the distribution of NP, M and NS1 between nuclear and cytoplasmic fractions following a

10 min pulse with radiolabel (Flawith, 1979; Flawith and Dimmock, 1979). However, in FP/R infected erythrocytes amounts of NP, M and NS1 in both cytoplasmic and nuclear fractions, following a 10 min pulse with radiolabel, were practically the same. After incubation of erythrocytes for 2 h in the absence of radiolabel the amounts of NP, M and NS1 were higher (2-fold for NP and M and 5-fold for NS1) in the nucleus than in the cytoplasm, suggesting some nuclear accumulation of these proteins. However, it is apparent from Fig. 24 that overall there is a close similarity between the levels of NP, M and NS1 in the nucleus with those in the cytoplasm. This was not observed in FP/BEL infected CEF cells (Flawith, 1979). Therefore, although the values shown in Fig. 24 are from only one experiment, it is tempting to speculate that there is little selectivity (with the exception of HA and presumably NA), over the migration of viral proteins into the erythrocyte nucleus. This seems to be true for the P proteins which migrate at the same rate into the nuclei of infected erythrocytes but at different rates into the nuclei of CEF cells.

SECTION 4

The contribution of immature and mature cells in virus infections of erythrocytes from 13 day-old chicken embryos

1. Introduction

Lucas and Jamroz (1961) found a significant proportion (approximately 14%) of immature cells, comprising mainly of mid- and late-polychromatic erythrocytes, in the erythrocyte population of 13 day-old chicken embryos. As already described (see General Introduction) the metabolic activity of erythrocytes decreases rapidly with maturation. Intuitively, one would expect a positive correlation between metabolic activity and suitability as a virus host cell. Therefore, it is possible that immature erythrocytes are the major producers of viral polypeptides and in the case of productive infections, the major producers of progeny virus. Against this are the data from fluorescent antibody staining which suggested that in infections with fowl plague, viral protein synthesis occurs in the entire erythrocyte population (see General Introduction). However, we examined in more detail the roles played by immature and mature cells in virus infections of erythrocytes. The approach adopted was to separate cells by discontinuous BSA density gradients (see Materials and Methods). This technique is based on the observation that the average buoyant density of avian erythrocytes appears to increase as they mature (Kabat and Attardi, 1967).

2. Results

Characterisation of the cells from each BSA gradient fraction

The details of preparation, centrifugation and harvesting of BSA gradients are described in Materials and Methods. However, as much of the following section is concerned with description of the various fractions obtained from BSA gradients Fig.26 is included as a reminder of the identity of these fractions.

a. The number of cells obtained from each fraction

Table 8 shows the distribution of cells in each fraction of a BSA gradient following centrifugation. The majority of cells were found in fraction 5 (86%) and in fraction 4 (about 11%).

Leucocytes were disregarded because of their low number (a maximum of 5×10^4 per 10^9 blood cells) and because they tend to become widely distributed throughout the BSA gradient after centrifugation (Attardi *et al.*, 1970; Appels *et al.*, 1972).

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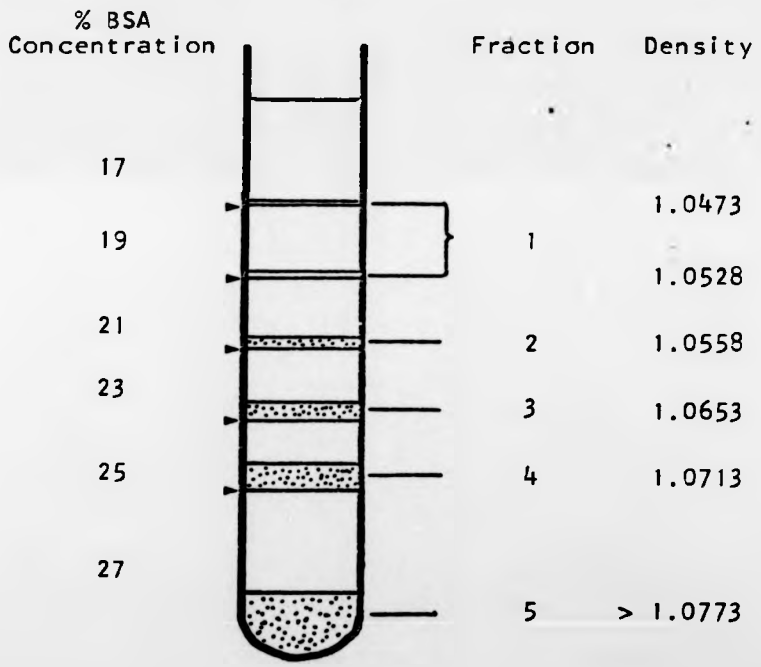
Fig. 26

The fractions harvested from a discontinuous BSA gradient (see Materials and Methods)



The hatching represents layers of cells which accumulate just above the concentration interfaces after centrifugation.

Table 8

Distribution of 13 day embryo blood cells after centrifugation through BSA gradient fractions

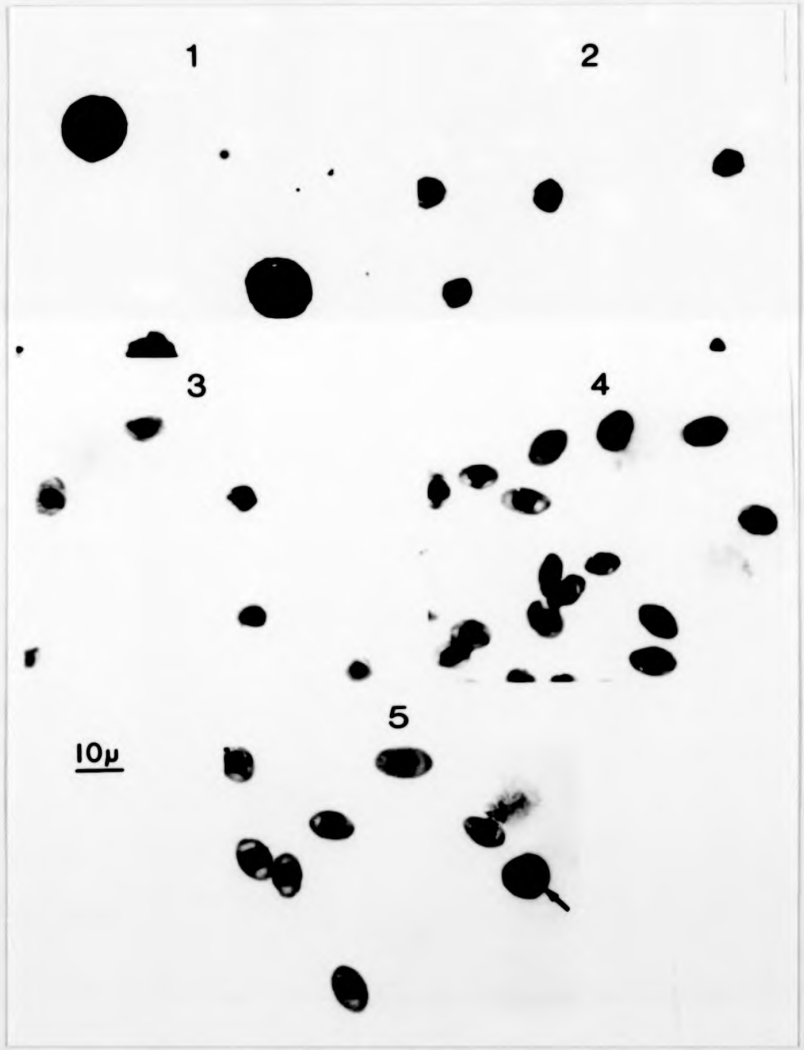
BSA Gradient Fraction	Cells/fraction	
	Number	Percentage
1	1.2×10^6	0.12 ± 0.07
2	1.9×10^6	0.19 ± 0.10
3	22.8×10^6	2.30 ± 0.30
4	111.0×10^6	11.20 ± 0.05
5	850.0×10^6	86.10 ± 0.30

The values shown in this table represent average figures obtained from 30 gradients. The percentage variation is shown in column B.

b. Morphology

Cells stained with May-Grumwald-Giemsa from each BSA gradient fraction are shown in Fig. 27.

Many of the cells found in fraction 1 (Fig. 27.1) were recognisable as erythroblasts according to the morphological criteria of Lucas and Jamroz (1961). These cells were larger than those of other fractions, were irregular in shape and possessed a granular cytoplasm. Their nuclei were seen as an open coarse network with some clumping of chromatin. The cells of fraction 2 (Fig. 27.2) were rounded and much smaller than the erythroblasts. The cytoplasm of these cells appeared homogenous rather than granular and stained blue with May-Grumwald-Giemsa (whereas cells containing large amounts of haemoglobin would stain orange). The cells of fraction 3 (Fig. 27.3) had the beginnings of an oval shape and a nucleus that was much more condensed than in the cells of fraction 2. The cytoplasm stained blue. Many of the cells of fraction 4 (Fig. 27.4) were oval and the cytoplasm of approximately 15% stained orange indicating the presence of large amounts of haemoglobin. The majority of cells in fraction 5 (Fig. 27.5) were oval and all had an orange cytoplasm when stained. However, the depth of this orange colour varied, suggesting the haemoglobin content of some cells was higher than in others. Such variation was not seen in erythrocyte preparations


1
2
3
4
5
10μ

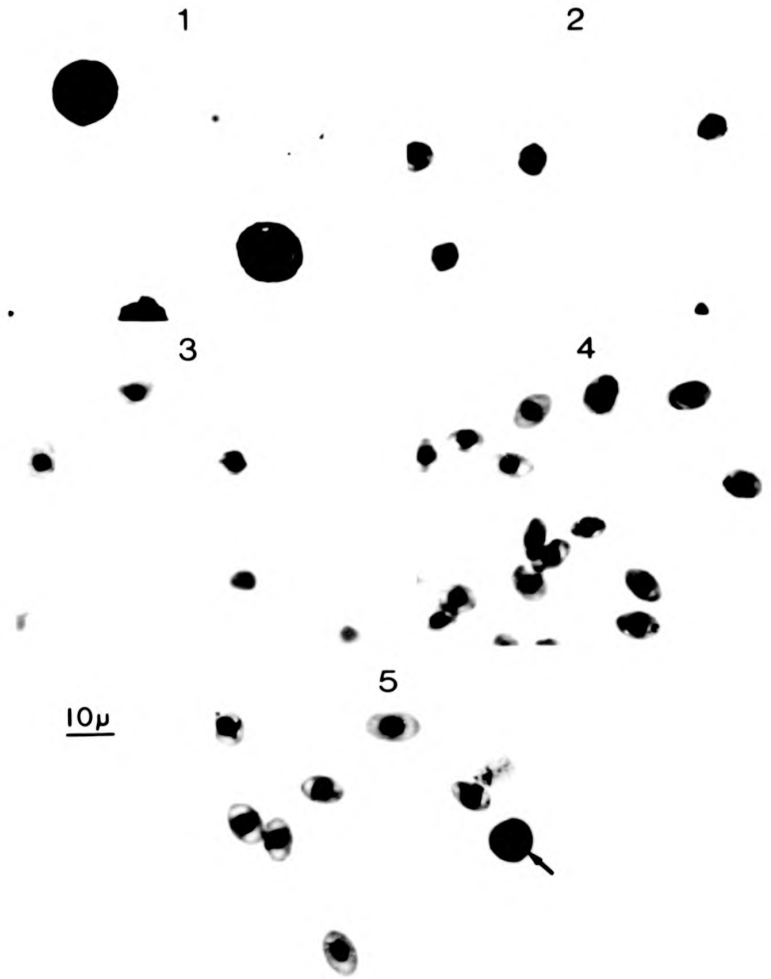

1
2
3
4
5
10μ

Fig. 27

Micrographs of cells from each fraction of a BSA gradient

1 corresponds to cells harvested from BSA gradient fraction 1, 2 to cells from fraction2and so on.

from adult birds (data not shown). The larger cell arrowed in Fig. 27.5 was an erythrocyte of the primary series as judged by its more rounded shape and larger size.

A precise classification of the cells from each gradient fraction is not possible since avian erythropoiesis is a continuous process. Therefore, many cells are in a transitional state between one developmental stage and the next. However, the predomonant erythroid cell of fraction 1 is the erythroblast. Fractions 2 to 4 contain polychromatic erythrocytes with fraction 2 having a preponderance of the earliest of these cells and fraction 4 having a higher proportion of cells nearing maturity. The majority of cells in fraction 5 have reached maturity as judged by their morphology and by the presence of haemoglobin. The proportion of cells in fraction 5 agrees with the number of mature erythrocytes that has been reported in the literature for embryos of this age (Romanoff, 1960).

c. Metabolic activities

A comparison of the metabolic activities of the cells from each BSA gradient fraction was made to augment the morphological characterization. Here metabolic activity refers to the ability of the cells to synthesize DNA, RNA and proteins. This ability was estimated by measuring incorporation of radioactive precursors into TCA precipitable material.

i) Incorporation of ^{3}H-thymidine

Figure 28 shows the abilities of the cells from each gradient fraction to incorporate ^{3}H-thymidine into TCA precipitable material. With each fraction incorporation of radioactive precursor was approximately linear with respect to time. However, there was a progressive reduction in the amounts of ^{3}H-thymidine incorporated from fraction 1 through to fraction 5. This is perhaps emphasized by the percentages shown in Table 9. Fraction 1 was given an arbitrary value of 100% and the levels of incorporation in other gradient fractions were calculated relative to this.

ii) Incorporation of ^{3}H-uridine

Incorporation of ^{3}H-uridine by cells from each gradient fraction is shown in Fig. 29. Once again incorporation was linear with respect to time and there was a progressive decrease from fraction 1 through to fraction 5. However, this decrease appeared more marked than with ^{3}H-thymidine incorporation particularly in the drop in activity between fractions 4 and 5, i.e. the majority of the 300-fold decrease in ^{3}H-uridine uptake from fractions 1 to 5 was contributed by the cells of fraction 5. The size of this decrease may, in part, be due to the rapid turnover of RNA that is known to occur in mature erythrocytes (Attardi et al., 1970; Zentgraf et al., 1975).

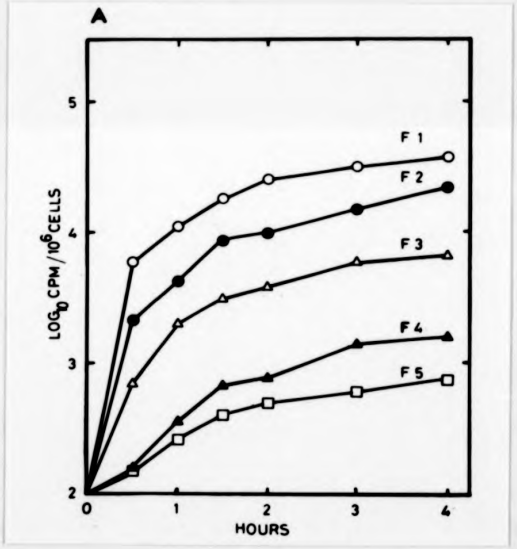

A
5
4
3
2
0
1
2
3
4
HOURS
LOG10 CPM / 10^6 CELLS
F 1
F 2
F 3
F 4
F 5

Fig. 28

Incorporation of ^{3}H-thymidine into TCA precipitable material in cells from each BSA gradient fraction

Fractions 1 to 5 were harvested from a total of 6 BSA gradients. The cells were washed four times with maintenance medium and counted. Cells from fractions 1 and 2 were resuspended in maintenance medium at 2×10^{6} cells/ml while the other 3 fractions were resuspended at 10^{7} cells/ml. All five fractions were incubated for 1 h at 37°C before the cells were pelleted, the medium removed and resuspended in Earles/20 mM HEPES/1% plus ^{3}H-thymidine (40 µCi/10^{7} cells). Duplicate 250 µl samples were taken from each fraction at the time of addition of the radioactive precursor and then at intervals for up to 4 h. TCA precipitated radioactivity was determined as described in Materials and Methods and expressed per 10^{6} cells.

Table 9

The relative incorporation of radioactive thymidine, uridine and leucine into cells of each BSA gradient fraction after incubation of 3 h. (Data from Figs. 28, 29, 30)

	% Incorporation into TCA↓ material		
Fraction	^{3}H-Thymidine	^{3}H-Uridine	^{3}H-Leucine
1	100	100	100
2	48	32	62
3	19	9	46
4	5	2	27.5
5	2	0.3	4.0

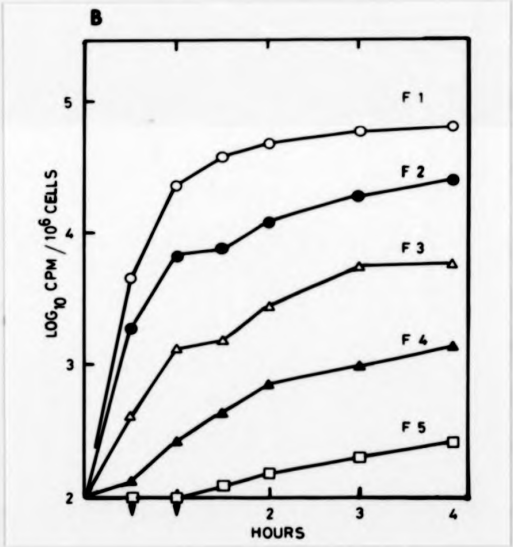

B
F 1
F 2
F 3
F 4
F 5
LOG_{10} CPM / 10^6 CELLS
5
4
3
2
2
3
4
HOURS

Fig. 29

Incorporation of ^{3}H-uridine into TCA precipitable material in cells from each BSA gradient fraction

The experimental protocol described in Fig. 28 was used except that cells were radiolabelled with ^{3}H-uridine (50 μCi/10^{7} cells). TCA precipitated radioactivity (Materials and Methods) is expressed as counts per 10^{6} cells.

An experiment to establish this point might be to pulse label erythrocytes from fraction 5 with ^{3}H-uridine and see if this can be chased from TCA precipitable to TCA soluble material, indicating synthesis and subsequent degradation of RNA. Similar experiments with the cells of other BSA gradient fractions would serve for comparison.

iii) Incorporation of ^{3}H-leucine

As with radionucleotides, incorporation of ^{3}H-leucine decreased as the bouyant density of the cells increased. However, the cells from fraction 1 gave rather variable results. In the experiment shown in Fig. 30 their rate of ^{3}H-leucine incorporation was apparently lower than cells of fractions 3 and 4 until after the first hour of incubation. In a second, similar experiment (data not shown) incorporation of ^{3}H-leucine in cells from fraction 1 was practically the same as that in cells from fraction 4. The reason for this variation was felt to be a reflection of the relatively small numbers of cells present in fraction 1.

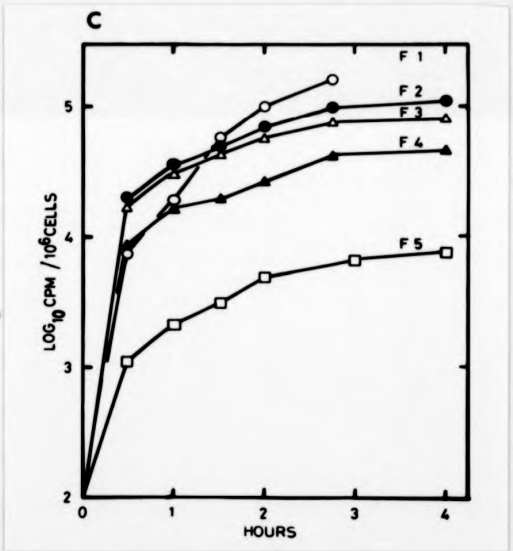

C
F 1
F 2
F 3
F 4
F 5
LOG_{10} CPM / 10^6CELLS
HOURS
2
3
4
5
0
1
2
3
4

Fig. 30

Incorporation of ^{3}H-leucine into TCA precipitable material in cells from each BSA gradient fraction

The experimental protocol described in Fig. 28 was used except that cells were radiolabelled with ^{3}H-leucine (10 μCi/ml) and samples were boiled in 10% TCA (Materials and Methods) before counting. TCA precipitable radioactivity is expressed as counts per 10^{6} cells.

d. Comparison of the metabolic activities of cells from BSA gradient fractions with CEF cells

The ability of cells from each gradient fraction to incorporate radioactive thymidine, uridine and leucine into TCA precipitable material was compared to that of an equivalent number of CEF cells (Table 10).

DNA synthesis in fraction 1 was 80% of the level in CEF cells. This decreased in cells of increasing density so that by fraction 5 it was just under 2%. The table also serves to illustrate, again, the very low levels of ^{3}H-uridine incorporation by cells of fraction 5. This is some 500-fold lower than in CEF cells.

Levels of ^{3}H-leucine incorporation by cells of each gradient fraction were generally high relative to CEF cells. Even fraction 5 appeared to be quite active (7% of the CEF cell level) in protein synthesis.

e. The contribution of each fraction to the metabolic activity of blood

If incorporation of the radioactive precursors is recalculated on the basis of the proportion that each fraction has in blood then a quite different picture emerges. Thus as described above the cells of fraction 5 incorporate amounts

Table 10

Comparison of metabolic activities of cells from each BSA gradient fraction with CEF cells all labelled for a 3 h period

	% Incorporation into TCA↓ material		
	^{3}H-Thymidine	^{3}H-Uridine	^{3}H-Leucine
CEF cells	100	100	100
Fraction			
1	81.0	54.0	160.0
2	40.0	17.0	100.0
3	16.0	5.0	79.0
4	4.0	1.0	45.0
5	1.6	0.2	7.0

of radioactive precursor lower than the cells of other gradient fractions. However, because fraction 5 comprises some 86% of the total number of cells it is the largest contributor to the metabolic activity of blood (Table 11).

As can be seen from Table 11, cells of other gradient fractions also make significant contributions to the metabolic activity of blood and may have influenced the results on viral protein synthesis reported in previous sections. The majority of experiments described in previous sections were on Ficoll separated, but otherwise unfractionated blood. Centrifugation of blood through 10% Ficoll reduced the number of cells in fractions 1 and 2 by approximately 10-fold but it had little effect on the relative numbers of cells in other fractions. The low contribution of fractions 1 and 2 to the metabolic activity of blood plus the further reduction caused by centrifugation through Ficoll, make it unlikely that these fractions would have played any part in previous results. However, the abilities of fractions 3, 4 and 5 to support virus protein synthesis have still to be accounted for.

Table 11

The contribution of each fraction to the metabolic activity in blood of 13 day old embryos

Fraction	Proportion in blood	% Contribution to the metabolic activity of 13 day embryo blood		
		^{3}H-Thymidine	^{3}H-Uridine	^{3}H-Leucine
1	0.12	4.0	14.0	3.0
2	0.19	6.0	7.0	2.3
3	2.3	8.0	24.0	9.0
4	11.2	30.0	21.0	40.0
5	86.1	52.0	34.0	48.0

The figures for the levels of radioactivity incorporated into TCA‡ material 3 h after addition of the radiochemicals were adjusted to take into account the total number of cells in the blood of 13 day old embryos.

The ability of cells of each BSA gradient fraction to support virus protein synthesis

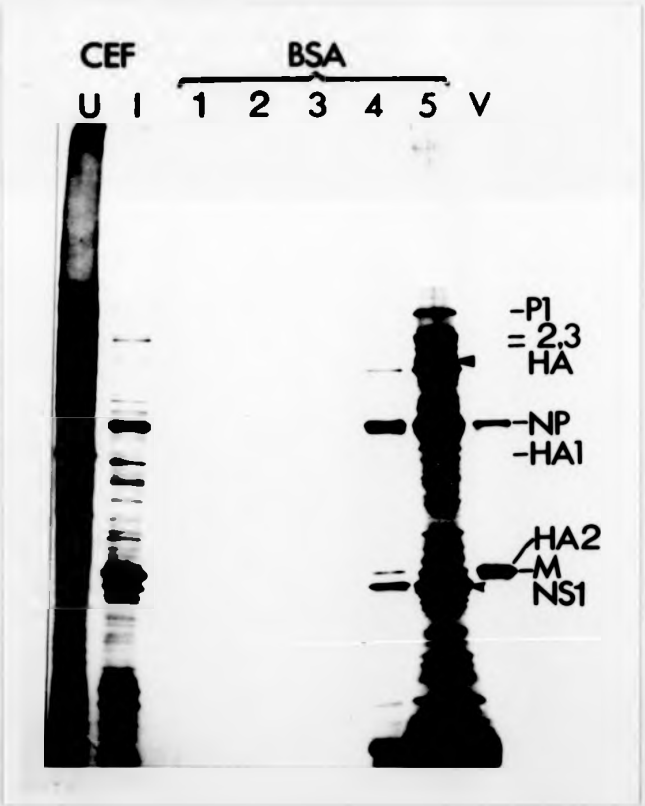
Cells from each fraction were infected with FP/R, radiolabelled with ^{35}S-methionine and loaded onto a polyacrylamide gel according to their proportion in 13 day old embryo blood. Only fractions 4 and 5 incorporated significant radioactivity (Fig. 31). Therefore, the results of PAGE analysis shown in previous sections should be interpreted as being due to the mature cells of fraction 5 with some contribution from the less well differentiated cells of fraction 4.

Virus protein synthesis in cells from each BSA gradient fraction

Samples of cells from each BSA gradient fraction were either infected with FP/R (moi 30 PFU/cell) or mock-infected with allantoic fluid. Figure 32 shows analysis of this experiment by PAGE. The polyacrylamide gel was loaded where possible on the basis of equal TCA-precipitable counts.

1. Mock-infected cells

As erythroid cells increase in density (i.e. as they mature) changes can be seen in the pattern of proteins synthesized:


CEF
BSA
U I 1 2 3 4 5 V
-P1
= 2,3
HA
--NP
-HA1
HA2
M
NS1

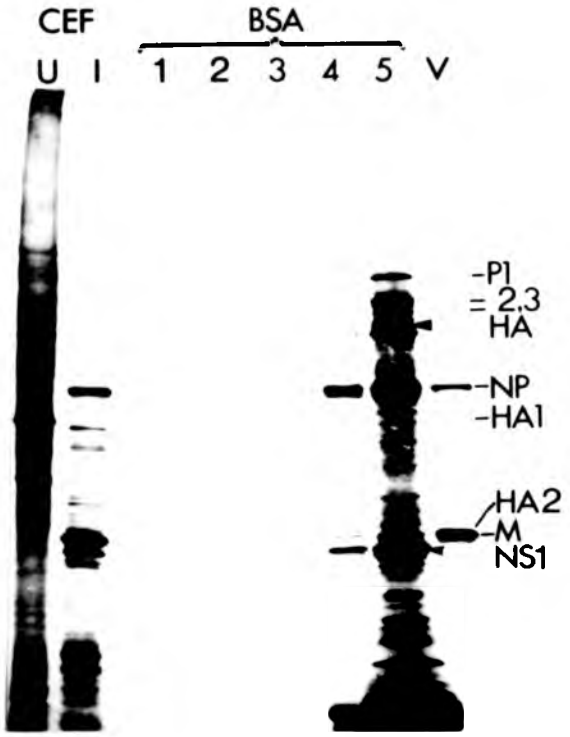

CEF
BSA
U I 1 2 3 4 5 V
-P1
= 2,3
HA
-NP
-HA1
HA2
-M
NS1

Fig. 31

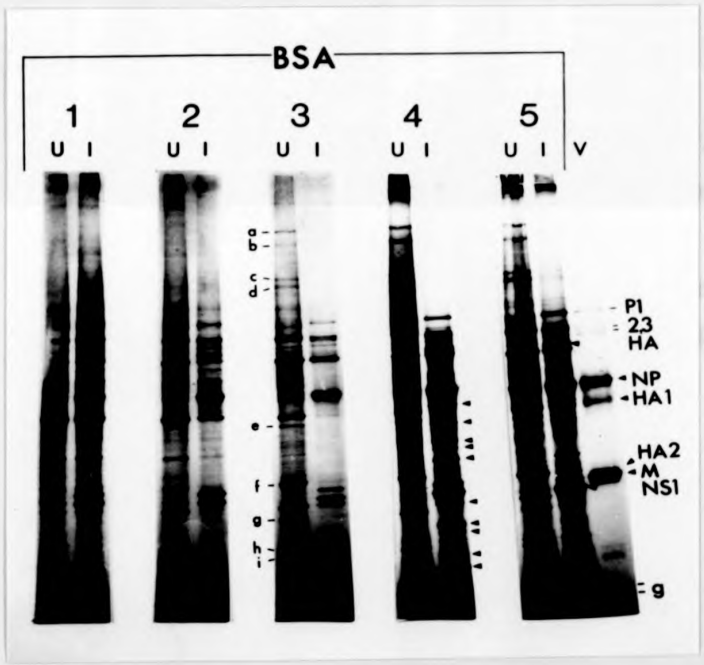
The ability of cells of each BSA gradient fraction to support virus protein synthesis

Cells from each gradient fraction were infected with FP/R (30 PFU/cell) and radiolabelled with ^{35}S-methionine (100 μCi/10^7 cells) 6 to 6.5 h post-infection. Samples from each fraction were loaded onto a polyacrylamide gel according to their relative proportions in 13 day embryo blood. Infected (i) and non-infected (ni) CEF cells and ^{35}S-methionine-labelled virions (V) were included as markers.

a) Globin synthesis was first detected in the cells of fraction 2 and was observed to increase markedly in the cells of fraction 3. This comparison was justified since mock-infected fractions 2 and 3 contained equal cell numbers. Whether a further increase in globin synthesis occurred in fraction 4 could not be determined by eye since tracks U3 and U4 were loaded as closely as possible with equal radioactivity and not with equal numbers of cells.

b) Other polypeptides appear as erythroid cells mature. Four high molecular weight (> 120,000) proteins (labelled a to d) were present in the cells of fractions 3, 4 and 5 but not in those of fractions 1 and 2. Also seen in fractions 3, 4 and 5 but not in 1 and 2 were a series of polypeptides labelled e to i with molecular weights ranging from 47,000 to 10,700. These were clearly visible in fractions 3 and 4 but of reduced relative intensity in fraction 5 (the fraction 5 track was loaded with four times the number of cells as the Fraction 4 track).

During erythrocyte maturation the synthesis of none of the existing proteins is shut off unilaterally, with the possible exception of actin (labelled ac in Fig. 32) synthesis which appears to be depressed relatively more than other proteins. Instead there appears to be a generalized reduction in the


BSA
1
2
3
4
5
U I
U I
U I
U I
U I V
a
b
c
d
e
f
g
h
i
P1
23
HA
NP
HA1
HA2
M
NS1
g

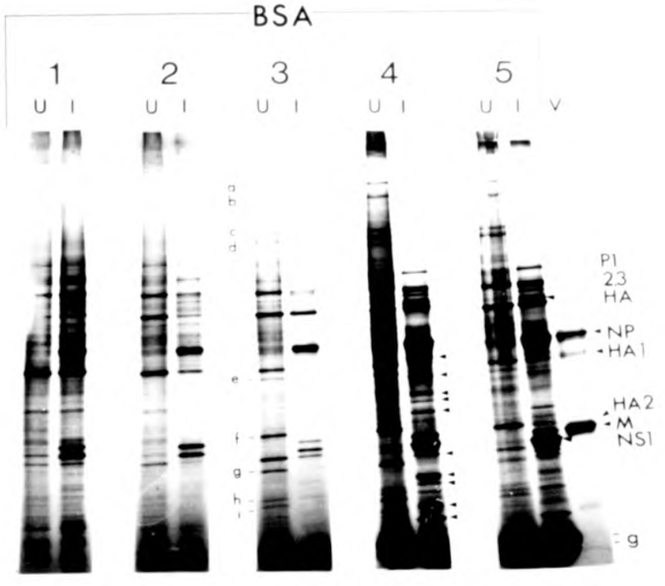

BSA
1
2
3
4
5
U I
U I
U I
U I
U I V
a
b
c
d
e
f
g
h
i
P1
23
HA
NP
HA1
HA2
M
NS1
g

Fig. 32

Virus protein synthesis in cells from each BSA gradient fraction

Cells from BSA gradient fractions 1 to 5 (F1 to F5) were infected (i) with FP/R or mock-infected (mi) and radio-labelled with ^{35}S-methionine (100 µCi/10^7 cells) 6 to 6.5 h p.i. Infected (i) and mock-infected CEF cells (mi) and ^{35}S-methionine labelled virions (V) were included as markers.

level of protein synthesis. For instance U5 was loaded with seven times the number of cells as U1. Similar observations concerning protein synthesis in erythrocyte maturation have been reported previously (Madgwick et al., 1972).

Thus, in summary erythrocyte maturation involves the synthesis of a few new proteins but at the same time the general level of protein synthesis is reduced.

2. FP/R infected cells

The cells of all gradient fractions were capable of supporting the synthesis of FP/R proteins (Fig. 32). The viral proteins detected in each fraction were P1, P2, P3, NP, M and NS1. In 4 + FP/R and in 5 + FP/R there was a diffuse band not present in the uninfected controls with a MW of ~76,000. This protein band probably is uncleaved HA. Longer exposures of the autoradiogram (data not shown) show that this protein was probably present in the other fractions. The amounts of M synthesized relative to NS1 were roughly equivalent in the cells of fractions 1 and 2. However, in the cells of fractions 3, 4 and particularly in 5 the amounts of M relative to NS1 were reduced. Possible reasons for this will be discussed later.

Also seen in fraction 4 were at least five protein bands (arrowed ; MW from 42,000 to 29,000) migrating in the region between NP and M. The majority of these protein bands were also present in fraction 5 but probably not present in other fractions since longer exposures of the gel to autoradiography (data not shown) failed to reveal any such bands above the host cell background. The first of these protein bands below NP has a MW of 42,000 and could possibly be HA1. However, its rate of migration is slightly slower than HA1 from purified virions. The other protein bands have not be reported previously (Lazarowitz et al., 1971; Skehel, 1972; Inglis et al., 1976; Lamb and Choppin, 1976; Ritchie et al., 1976). The origin of these protein bands is not clear. They may be cleavage products of larger viral proteins arising either inside the cell during infection or by some exogenous protease activity during preparation of samples for PAGE. Alternatively, they may be host proteins whose appearance was in some way promoted by FP/R infection.

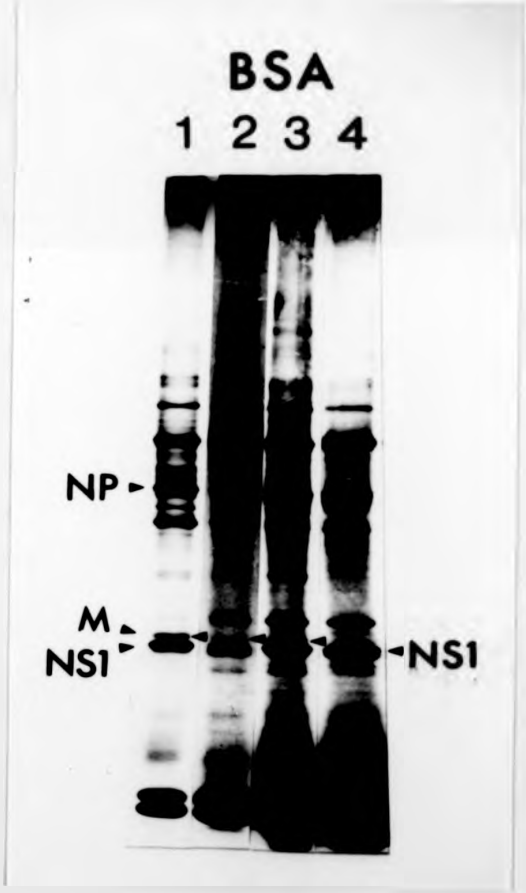
Present in fractions 4 and 5 were at least five bands (arrowed in Fig. 32) migrating in the region below NS1. We have also seen similar protein bands in FP/R infected CEF cells (Cook et al., 1979). These have MWs ranging from ~22,000 to ~11,000. Lamb et al. (1978) found protein bands with a similar MW range in WSN-infected CEF cells and showed

by tryptic peptide analysis that all but one, designated NS2, were cleavage products of NS1.

Infection of cells from each BSA gradient fraction with NWS

Previous experiments (see Section 2) had shown that erythrocytes did not permit the synthesis of M protein when infected with the influenza strain NWS. However, these experiments were performed on Ficoll separated erythrocytes and, as already pointed out, under these conditions only proteins made by cells late in the erythropoietic series could be detected on polyacrylamide gels. Therefore, the possibility existed that the small proportion of cells representing the earlier part of the developmental sequence might permit the expression of M protein by NWS.

Figure 33 shows the results of PAGE analysis after infection of the cells of each gradient fraction with NWS. Both M and NS1 proteins were seen in fractions 1, 2 and 3 but not in fraction 4. The cells of fraction 5 labelled poorly in this experiment and so were not included. Furthermore, from fractions 1 to 3 there appears to be a progressive reduction in the amount of M relative to NS1. Therefore, it is concluded that cells early in the erythrocyte series allow the expression of NWS M protein.


BSA
1 2 3 4
NP
M
NS1
NS1

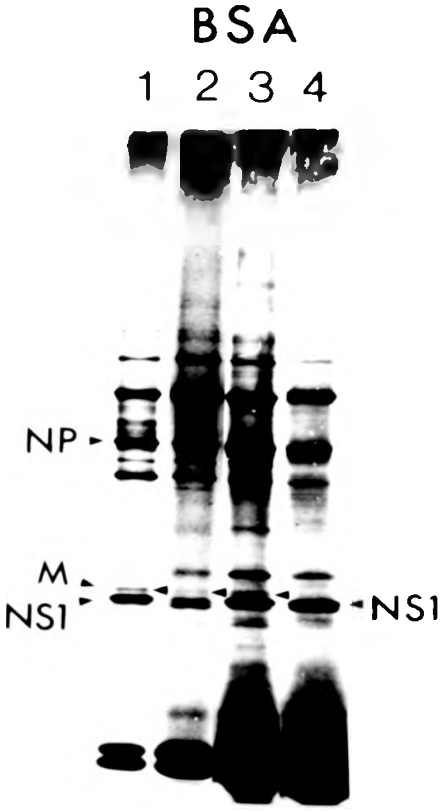

BSA
1 2 3 4
NP
M
NS1
NS1

Fig. 33

PAGE of A/NWS proteins synthesized by the cells of each gradient fraction

Cells from each gradient fraction were infected with A/NWS (30 E ID_{50}/cell) and incubated for 1 h at 37°C. Virus was removed, the cells resuspended in maintenance medium, incubated at 37°C, and radiolabelled at 6 to 6.5 h p.i. with ^{35}S-methionine (100 µCi/10^7 cells).

Markers were provided by infected (i) and non-infected (ni) CEF cells.

Multiplication of NDV in mature and immature erythrocytes

In Section 1 it was shown that NDV replicated in Ficoll purified erythrocytes although the titre was only 1 PFU/200 cells. An explanation for this low titre would be that virion production was confined to certain of the immature cells in the erythrocyte population. This suggestion was tested by separating mature from immature erythrocytes using BSA gradients and infecting them with NDV. Immature erythrocytes were regarded as those cells sedimenting in fractions 1 to 4 and mature erythrocytes as those cells sedimenting in fraction 5.

Immature erythrocytes, mature erythrocytes and erythrocytes that had been centrifuged through 10% Ficoll were infected with NDV. After 1 h at 37°C cells were disaggregated with trypsin and treated with neutralizing antiserum to reduce levels of inoculum virus (Matierals and Methods). Samples of cells and maintenance medium were disrupted by sonication at intervals after infection and infectivity measured by plaque assay on CEF monolayers. The results suggest (Fig. 34) that it is the immature rather than the mature erythrocytes that produce the majority of progeny NDV. The 3-fold rise in infectivity in mature erythrocytes represents only 1 PFU/12,500 cells. However, the population of cells from gradient fractions 1 to 4 contained 7-fold more mid- and

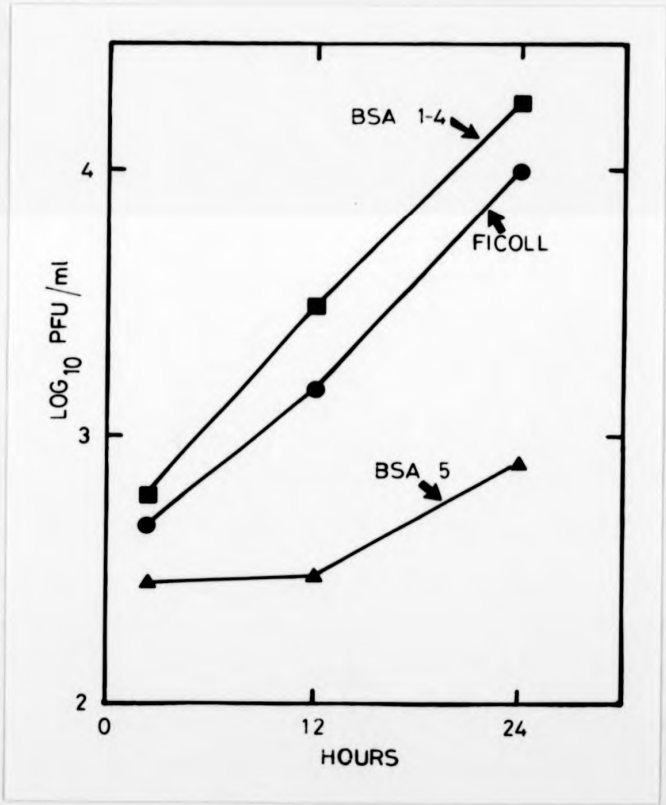

BSA 1-4
FICOLL
BSA 5
4
3
2
0
12
24
HOURS
LOG10 PFU /ml

Fig. 34

Multiplication of NDV in mature and immature erythrocytes

Two 1 ml samples of 10^7 cells were taken from immature (pooled BSA gradient fractions 1 to 4) and mature (from BSA gradient fraction 5) erythrocytes at intervals after infection with NDV and assayed for infectious progeny virus production by plaque assay on CEF monolayers.

late-polychromatic erythrocytes and 70-fold (since centrifugation of erythrocytes through Ficoll reduces the number of cells in fractions 1 and 2 by approximately 10-fold) more erythroblasts and early-polychromatic erythrocytes than Ficoll purified erythrocytes. Therefore, erythrocytes from fractions 1 to 4 would be expected to show a larger than 2-fold increase over Ficoll-purified erythrocytes in the level of progeny NDV produced. Furthermore, this preliminary experiment does not identify those erythrocytes which synthesize the greatest amounts of progeny NDV. Further experiments would require the cells of each gradient fraction to be infected separately and the results expressed on the basis of PFU/cell.

3. Discussion

Erythrocytes from 13 day old chicken embryos could be separated according to their state of differentiation by the use of discontinuous BSA gradients as previously described for the erythrocytes of adult birds by Appels et al. (1972), Williams (1972) and Longacre and Rutter (1977).

Five fractions were obtained from these gradients. By morphological criteria fraction 1 contained predominantly erythroblasts while fractions 2 to 4 contained polychromatic erythrocytes. Fraction 2 had a preponderance of early-polychromatic erythrocytes and fraction 4 a high proportion of cells nearing maturity. The cells of fraction 3 were in an intermediate state of differentiation. Morphologically mature erythrocytes were isolated from fraction 5.

Measurements of the ability of the cells of each fraction to incorporate radioactive macromolecular precursors into TCA precipitable material added some confirmation to the morphological classification. Since in general incorporation decreased as buoyant density (which as previously explained is related to the degree of maturity of erythrocytes (Kabat and Attardi, 1967)) increased.

Measurements of ^{3}H-thymidine incorporation into TCA precipitable material suggested that mature erythrocytes from 13 day old

embryos were synthesizing DNA. This was in contrast to a similar analysis on equivalent cells from adult chickens (data not shown) in which ^{3}H-thymidine was not incorporated in significant amounts, an analysis agreeing with previously published results (Williams, 1972; Longacre and Rutter, 1977). Thus it appears that mature erythrocytes from 13 day-old chicken embryos may be undergoing some residual DNA replication and/or repair synthesis.

Although erythrocytes from embryos contain some comparatively 'active' cells in terms of incorporation of radioactive substrates it is the most mature cells, simply because of their numbers, that are the major contributors to the metabolic activity of the erythrocyte population. In other words, in unfractionated erythrocytes, ^{3}H-thymidine and ^{3}H-leucine incorporation by the mature erythrocytes would account for approximately 50% of the total. However, the cells sedimenting in fraction 4 would account for approximately 30%. This suggested that the results shown by PAGE in previous sections should be interpreted as being due to mature erythrocytes with some contribution from late polychromatic erythrocytes. A suggestion confirmed by loading infected cells from each gradient fraction onto a polyacrylamide gel in the proportion that they occur in the blood.

In Section 1 it was reported that only rRNA synthesis could be detected in ^{32}P-labelled unfractionated erythrocytes. At first sight this appeared to be at variance with reports that circulating erythrocytes from adult chickens did not synthesize rRNA (Longacre and Rutter, 1977; Zentgraf et al., 1975). However, from Table 11 mature erythrocytes would contribute only 30% to the total incorporation of ^{3}H-uridine by an erythrocyte population from 13 day-old embryos. Thus the bulk of the rRNA synthesis observed in Section 1 is most likely due to immature erythrocytes.

PAGE analysis of proteins synthesized by uninfected cells from each BSA gradient fraction revealed the following information:

Globin synthesis was detected early (Fraction 2) in the erythropoietic cycle. This agrees with reports that a 9S mRNA, thought to be globin mRNA, can be detected at the early polychromatic erythrocyte stage (Attardi et al., 1970).

Certain polypeptides (labelled a to i in Fig. 32) present in fractions 3 to 5 were not detected in fractions 1 and 2. This suggested that during erythrocyte maturation there may be certain genes which are actually de-repressed. Clues to the identity of these polypeptides may come from consideration of those polypeptides known to be synthesized

by avian erythrocytes. Simply on a MW basis it is possible that proteins labelled f to i could be histones (MW 12,000-20,000). However, synthesis of histones, including the unique erythrocyte histone (H5) is virtually complete at the erythroblast stage (Appels et al., 1972). Ruiz-Carrillo et al. (1974) have reported that a protein of MW 50,000 is selectively retained throughout erythrocyte maturation. However, Jeter et al. (1976) have shown that this and other nuclear acidic proteins are also synthesized predominantly at the erythroblast stage. Therefore, it is unlikely that polypeptides a-i are acidic nuclear proteins.

Chan (1977) purified the plasma membranes of erythrocytes from different ages of chicken embryo (2.5 days to 18 days). The results show a correlation between the age of the embryo, and hence the proportion of mature erythrocytes, and the accumulation of at least six proteins in the erythrocyte plasma membrane. Three of these proteins had MWs of over 200,000 two had MWs of approximately 100,000 and one had a MW of 42,000.

Polypeptides (a) and (b) had MWs of approximately 200,000, polypeptides (c) and (d) had Mws of just over 100,000 while polypeptide (e) had a MW of 47,000. Therefore, it is possible that bands a to e correspond to plasma membrane proteins. However, although polypeptides (a) to (e) were present in

the cytoplasmic fraction of uninfected erythrocytes (data not shown) reference to Section 3 (Fig. 23) shows that polypeptides (a) and (b), but not (c) or (d) were also present in the nuclear fraction. Thus if (a) and (b) were associated with the plasma membrane they may also be associated with the nuclear membrane. A test of this would be to isolate and characterise the proteins in both the nuclear and plasma membranes. A technique for separation of these types of membrane from erythrocytes has been described by Zentgraf et al. (1971). As for the possible identity of polypeptides f to i little can be said at present.

An examination of the proteins synthesized by the cells of each gradient fraction after infection with FP/R demonstrated that erythrocytes could support the synthesis of viral proteins throughout their developmental sequence. However, in the cells of fractions 3, 4 and particularly in 5, the amounts of M relative to NS1 were reduced. This may be explained if it is assumed that the putative host factor required for M protein synthesis (see Section 2) becomes reduced either in activity or in amount during erythrocyte maturation. As a consequence of this reduction, relatively less M protein is synthesized.

Infection of the cells of each gradient fraction with NWS yielded a different situation in that M protein synthesis

was lost altogether by the late polychromatic stage. The implication of this being that NWS either requires a higher concentration of host factor than FP/R to synthesize M or that it uses a different host factor which is absent in late polychromatic erythrocytes.

At least five polypeptides (MW 42-29,000) could be seen migrating between NP and M in FP/R infected cells of fractions 4 and 5. These polypeptides had been observed in FP/R infected erythrocytes in previous experiments although not in FP/R infected CEF cells. There are several possible explanations of their origin. The first is that they are virus coded and are formed by proteolytic cleavage either within the cell or in subsequent sample preparation. Another alternative is that in mature or nearly mature erythrocytes defects in translation occur which cause incomplete viral polypeptides to be produced. Still other explanations are based on the five polypeptides being of host origin and somehow influenced by FP/R infection. For instance, FP/R may stimulate the synthesis of certain host proteins. Alternatively, it may cause certain high MW host proteins to be degraded or perhaps even cause premature termination in the translation of certain mRNAs. In Section 2 (Fig. 17) it can be seen that at 3.5 h p.i. polypeptides A to D disappear while at the same time point there are traces of additional polypeptides migrating between NP and M.

However, it would probably be necessary to compare the peptide maps of these five polypeptides with those of suitable host or viral protein candidates in order to establish their origin.

Also present in FP/R infected cells of fractions 4 and 5 were several polypeptides migrating below NS1. Although the same arguments apply to these as just described above, polypeptides of a similar molecular weight range have been observed in WSN infected CEF cells (Lamb et al., 1978) and in FP/R infected CEF cells (Cook et al., 1979). All but one of these polypeptides, designated NS2, have been found to be cleavage products of NS1 (Lamb et al., 1978).

On infection with NDV immature erythrocytes (pooled fractions 1 to 4) produced, perhaps not surprisingly more progeny virions per cell than mature erythrocytes. In Section 1 it was reported that erythrocytes centrifuged through Ficoll, but otherwise unfractionated would synthesize detectable amounts of progeny SFV and NDV but not of FP/R. In view of the fact that the majority of the progeny virions synthesized were from immature rather than mature erythrocytes it is possible that all erythroid cells are non-permissive for FP/R infection. To check this would require that the cells of each gradient fraction be infected with FP/R and assayed independently.

SECTION 5

Infection of mature erythrocytes from adult chickens with FP/R

1. Introduction

Erythrocytes from 13 day old embryos, which are regarded as mature by morphological characteristics and by haemoglobin content, still retain a measurable level of metabolic activity. For instance, incorporation of TCA-precipitable ^{3}H-leucine and ^{35}S-methionine or analysis by PAGE shows them to be synthesizing a large number of proteins at about 7% of the level found in CEF cells. There are indications that mature erythrocytes from adult birds are much less metabolically active (Lucas and Jamroz, 1961; Kabat and Attardi, 1967; Williams, 1972). From this point of view the erythrocytes from 13 day and adult birds might be regarded as different cell types. This notion was investigated by determining the extent to which mature erythrocytes from adult birds could support FP/R protein synthesis. The experiment described in Section 1 of infecting Ficoll-separated adult chicken blood cells, goes some way towards this goal. However, the results of this experiment could have been influenced by the small number (5%) of immature erythrocytes present in the blood of adult chickens after Ficoll separation. In order to obviate this critism the erythrocyte population from adult chickens was fractionated using discontinous BSA gradients as previously described (see Materials and Methods).

2. Results

Characteristics of the cells from each BSA gradient fraction

a) The proportion of cells obtained in each fraction

Table12 shows the distribution of cells in each fraction following centrifugation of adult chicken blood through a BSA gradient. There were more mature erythrocytes (95% of the total) than in 13 day old chicken embryo blood (see Table 12).

The proportion of leucocytes in adult chicken blood is about 0.6% (Olson, 1963). These cells were removed in subsequent experiments by centrifugation through 10% ficoll (Materials and Methods) before centrifugation on BSA gradients, a procedure which reduced the already low number of cells present in Fractions 1 and 2 (see Table 12), to a point where it was not practicable to work with them.

b) Morphology

The morphologies of the cells in each BSA gradient fraction were similar to those described for 13 day old embryo blood (data not shown) except that adult erythroid cells tended to be slightly smaller than their 13 day old embryo counterparts (Wignall and Dimmock, unpublished data).

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Table 12

The proportion of cells sedimenting in each fraction of a BSA gradient

BSA Gradient Fraction	A Adult Blood % of cells in each fraction	B 13 day old Embryo Blood % of cells in each fraction
1	0.03	0.12
2	0.10	0.19
3	0.14	2.3
4	4.6	11.2
5	95.2	86.1

Column A shows the proportion of cells sedimenting in each fraction following centrifugation of adult chicken blood in BSA gradients. For comparison column B shows the proportion of cells found in the same fraction after centrifugation of 13 day old embryo blood in BSA gradients.

c) Incorporation of ^{3}H-Uridine

Figure 35 shows the abilities of the cells from BSA gradient fractions 3, 4 and 5 to incorporate ^{3}H-uridine into TCA precipitable material. With each fraction incorporation of radioactive precursor was approximately linear with respect to time but as observed with blood from 13 day old embryos (see Section 4), there was a progressive reduction in incorporation as the cells increased in density. As a comparison, incorporation of TCA precipitable ^{3}H-uridine into mature (fraction 5 of a BSA gradient) 13 day old embryo erythrocytes was measured and proved to be less than in mature erythrocytes from adult birds (Fig. 35). This result was not expected considering the relative levels of ^{35}S-methionine incorporation (see Section 1). A further check measured ^{3}H-leucine incorporation in erthyrocytes from 13 day embryos and adult birds. The mature erythrocytes from embryos incorporated 40-fold more radioactivity after a 4 h labelling period (Fig. 36a) than mature erythrocytes from adult birds. The results of a parallel experiment measuring ^{3}H-uridine incorporation into the two ages of mature erythrocyte are shown in Fig. 36b, a result confirming that shown in Fig. 35. Thus although incorporation of ^{3}H-leucine into TCA precipitable material is lower in mature adult erythrocytes than in mature embryo erythrocytes, incorporation of ^{3}H-uridine is somewhat higher (33-fold after a 4 h labelling period).

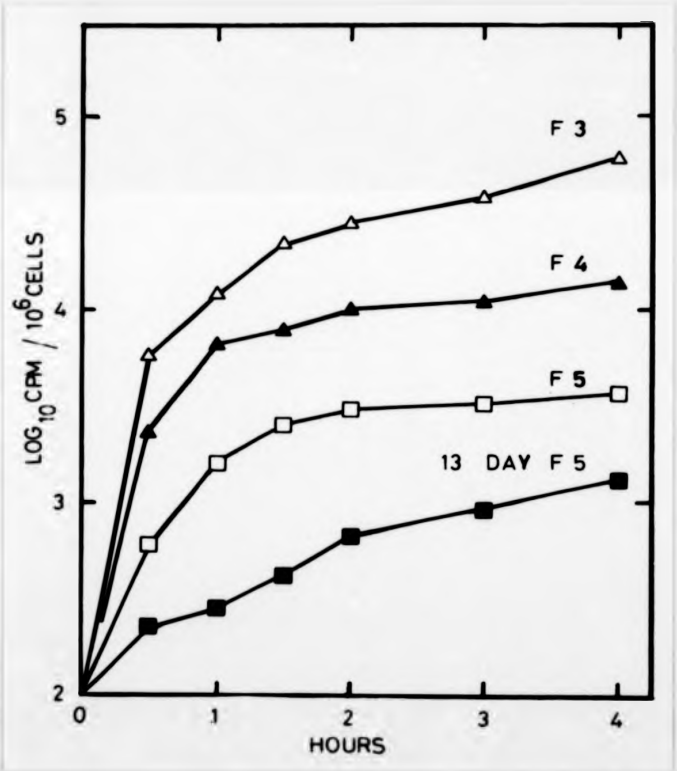
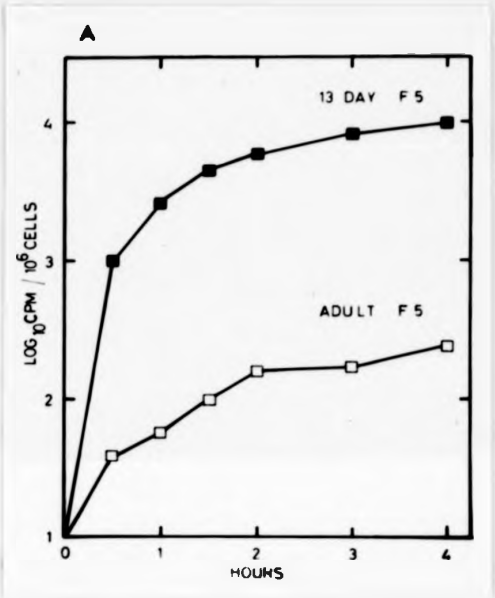

F 3
F 4
F 5
13 DAY F 5
LOG10CPM / 10^6CELLS
HOURS
5
4
3
2
0
1
2
3
4

Fig. 35

Incorporation of ^{3}H-Uridine into TCA precipitable material

Blood from an adult chicken was centrifuged through BSA gradients. Fractions 3, 4 and 5 were harvested as described in Materials and Methods. These were washed and resuspended in maintenance medium such that fraction 3 was at 10^6 cells/ml and fractions 4 and 5 were at 10^7 cells/ml. After incubation for 1 h at 37°C the cells were radiolabelled with ^{3}H-uridine (50 µCi/10^7 cells) in Earles/20mM Hepes/$\frac{1}{100}$ 199 (1 ml 199 per 100 mls of Earles saline). Samples were taken at the time of addition of radiochemical and at intervals for 4 h. TCA precipitable radioactivity was determined as described in Materials and Methods. Results are expressed as cpm/10^6 cells. As a comparison a similar, parallel experiment was performed with 13 day old embryo erythrocytes harvested from fraction 5 of a BSA gradient (labelled: 13D Fraction 5).


A
13 DAY F 5
ADULT F 5
LOG10CPM / 10^6 CELLS
HOURS
1
2
3
4
0
1
2
3
4

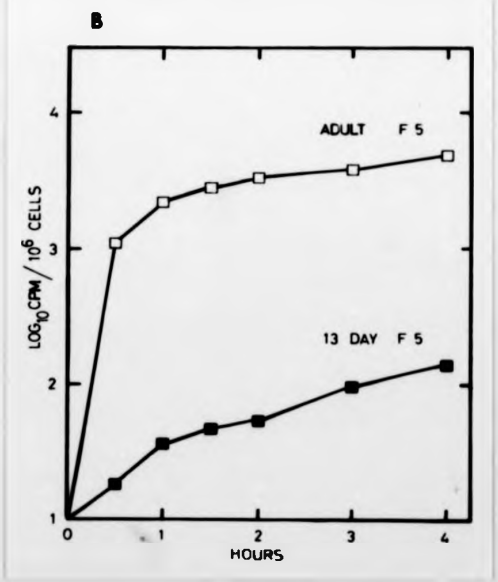

B
ADULT F 5
13 DAY F 5
LOG10CPM / 10^6 CELLS
HOURS
1
2
3
4
0
1
2
3
4

Fig. 36

Comparison of incorporation of ^{3}H-leucine and ^{3}H-uridine into TCA precipitable material in mature embryo erythrocytes and in mature adult erythrocytes

Mature erythrocytes (10^{7} cells/ml) from 13 day old chicken embryos and mature erythrocytes from an adult chicken were incubated in maintenance medium for 1 h at 37°C. Mature erythrocytes were harvested from fraction 5, cells were radiolabelled with ^{3}H-leucine (Fig. 36a) (10 µCi/10^{7} cells) or ^{3}H-uridine (Fig. 36b) (50 µCi/10^{7} cells) in Earles/20 mM Hepes/1% 199. Samples were taken at the time of addition of the radiochemical and at intervals for 4 h. TCA precipitable counts were determined as outlined in Materials and Methods.

d) TCA soluble and TCA precipitable radioactivity

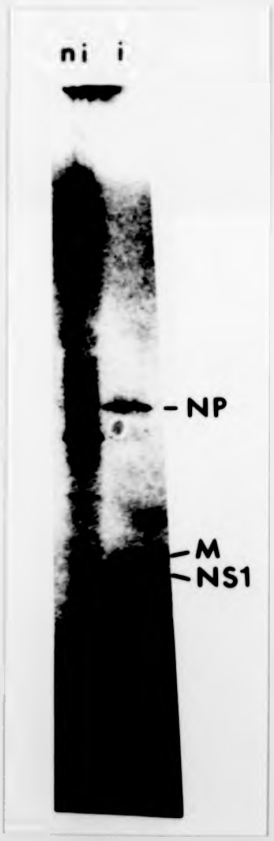
The results just described could be explained by the differential uptake of ^{3}H-uridine and this was investigated by determining TCA-soluble pools of radioactive-precursor. Table 13 shows TCA-soluble (determined by subtraction of TCA precipitable counts from total counts as outlined in Materials and Methods) and TCA precipitable radioactive counts, expressed in P moles, following labelling of mature erythrocytes with either ^{3}H-uridine (50 μCi/10^{7} cells) or ^{3}H-leucine (10 μCi/10^{7} cells). Samples were taken 2 h after addition of the radiochemical.

This experiment was only intended as a pilot study for one in which samples would be taken at intervals after the addition of radiochemical. Time did not permit this extended experiment to be carried out and so the results shown in Table 13 can only be regarded as preliminary. However, they suggest that despite differences in the levels of RNA synthesis the two ages of erythrocyte contain approximately the same amounts of 'free' ^{3}H-uridine. This can be contrasted to some extent to the situation with ^{3}H-leucine where embryo erythrocytes not only synthesize more protein than adult erythrocytes, but they also contain higher levels of 'free' ^{3}H-leucine. If these results could be verified they would argue against the explanation that the lower level of ^{3}H-uridine incorporation in embryo erythrocytes is due to some defect preventing the radiochemical entering the cell.

Table 13

Comparison of TCA soluble and TCA precipitable radioactivity in the mature erythrocytes of embryonic and adult chickens

	^{3}H-Uridine		^{3}H-Leucine	
	Adult p moles/ 10^6 cells	13 day Embryo p moles/ 10^6 cells	Adult p moles/ 10^6 cells	13 day Embryo p moles/ 10^6 cells
TCA Soluble	0.7	0.6	0.1	0.7
TCA Precipitable	0.1	0.01	0.05	1.6


ni i
NP
M
NS1


ni i
NP
M
NS1

Fig. 37

PAGE of proteins synthesized in FP/R-infected mature erythrocytes from an adult chicken

FP/R infected (i) or non-infected (ni) mature erythrocytes from an adult chicken were radiolabelled with ^{35}S-methionine as described in the text.

PAGE analysis of FP/R infected mature erythrocytes

Adult chicken blood, previously centrifuged through 10% ficoll, was fractionated into different cell types by the use of a discontinuous BSA gradient. Cells from fraction 5 were washed, infected with FP/R (30 PFU/cell) and radiolabelled with ^{35}S-methionine (150 μCi/10^7 cells) 6 to 6.5 h post-infection. The viral proteins NP, M and NS1 (Fig. 37) could be detected in the infected cell sample suggesting that even mature erythrocytes from adult birds could support the synthesis of fowl plague viral proteins.

3. Discussion

As previously reported (Williams, 1972; Appels et al., 1972; Longacre and Rutter, 1976) erythrocytes from adult chickens could be separated according to their state of differentiation by discontinuous BSA gradients. Approximately 95% of the erythrocytes were classified as mature, however they were found to incorporate less ^{3}H-leucine (40-fold) but, surprisingly, more ^{3}H-uridine than mature erythrocytes from 13 day old embryos. Thus, compared to the process in the embryo, erythropoiesis in the adult may involve a more stringent reduction in protein synthesizing capacity but

less of a reduction in RNA accumulation. Indeed, comparison of Fig. 35 of this section with Fig. 29 of Section 4 indicates that RNA accumulation may also be higher in those adult erythrocytes sedimenting in gradient fractions 3 and 4 than in equivalent erythrocytes from 13 day old embryos. (Experiments detailed in Fig. 29 Section 4 and Fig. 35, Section 5 were carried out using similar conditions but not in parallel). It is not clear whether these results reflect differences in functional requirements between erythrocytes of the 13 day old embryo and those of the adult or whether embryo erythrocytes derived mainly in the yolk sac (Lucas and Jamroz, 1961; Olson, 1963; Nigon and Godet, 1976) undergo slightly different maturational events from those of the adult which are derived from the bone marrow (Lucas and Jamroz, 1961; Olson, 1963; Godet, 1974; Nigon and Godet, 1976).

Infection of mature erythrocytes from adult chickens with FP/R demonstrated that even these fully differentiated cells could support viral protein synthesis. Furthermore, M protein synthesis was detected in addition to that of NP and NS1 implying that the mature erythrocytes of the adult bird still retain sufficient of the necessary host factor(s) to allow M protein expression.

Conclusions

The results presented here show that chicken erythrocytes, whether isolated from the embryo or from the adult bird, can support the synthesis of all the major FP/R proteins with a time course of appearance similar to that observed in CEF cells. However, despite this FP/R infection of chicken erythrocytes is apparently abortive.

Infection of erythrocytes with human strains of influenza virus produces a different situation in that no detectable viral M protein is synthesized. However, this defect can be rectified by co-infection with an avian influenza strain; a finding which may highlight the existence of a host-cell/virus interaction necessary for the expression of M protein.

In contrast to infection with influenza viruses infection of erythrocytes with either SFV or NDV leads to the production of some progeny virions. However, the titres were low and the synthesis seemed to be mainly confined to immature erythrocytes. Whether the capacity of immature erythrocytes to replicate certain viruses has any relevance in the disease process remains to be determined.

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Infection of Chicken Erythrocytes with Influenza and Other Viruses

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Chicken erythrocytes can be infected by the fowl plague (Rostock) strain (FP/R) of influenza type A, Newcastle disease virus (NDV), and Semliki Forest virus (SFV). Only NDV and SFV produced infectious progeny, albeit at low levels. Infection by FP/R was monitored by de novo synthesis of viral proteins, and the proteins synthesized could be identified by comparison with infected chicken fibroblast cells. FP/R synthesized far greater amounts of viral protein than did NDV or SFV.

In our earlier work (18, 29) we investigated chicken erythrocyte nuclei which are metabolically dormant (2, 13, 14, 27), to see if they could provide those nuclear functions which are necessary for the intracellular synthesis of influenza virus-coded components (4, 12, 17, 24, 28, 30). We introduced the erythrocyte nuclei into cultured cells which had been enucleated functionally with actinomycin D (18) or physically (29) and found that such reconstituted cells could support some viral macromolecular synthesis when infected with an influenza virus. This raised the possibility that viruses might be able to multiply in erythrocytes themselves; evidence to support this idea is provided by observations that erythrocytes are capable of pinocytosis of influenza viruses (5).

The association of viruses and erythrocytes during infections of animals has been reported for members of a number of different virus groups (see reference 11), and Colorado tick fever virus has been one of the most thoroughly studied (11, 33). However, these authors conclude that Colorado tick fever virus infects erythropoietic cells, which results in the presence of virions in erythrocytes. In this report we describe the infection of chicken erythrocytes in vitro by influenza and other viruses and the subsequent synthesis of virus-coded components or infectious virus. We understand that this is the first instance of virus multiplication in erythrocytes infected in vitro, and we believe that this will be of interest to those investigating both pathogenesis and virus-cell interactions.

MATERIALS AND METHODS

Cells. Chicken embryos used in these experiments were from a hybrid flock derived from a Light Sussex female and a C020 male. Eggs were obtained from a commercial breeder (Lockaley Ltd., Nuneaton, England).

Avian erythrocytes were obtained from 13-day-old chicken embryos by cutting the allantoic blood vessels and allowing blood to drain into the allantoic fluid for 10 min. This fluid was removed from the egg, and erythrocytes were collected by centrifugation at 500 × *g* for 10 min and washed twice in Alsever solution.

Monolayers of primary chicken embryo fibroblast (CEF) cells were prepared in 5-cm petri dishes (32).

Viruses. We used influenza virus A/FPV/Rostock/34 (Havl N1) (FP/R), Newcastle disease virus (NDV) strain Texas, and Semliki Forest virus (SFV). FP/R stocks were grown in 11-day embryonated chicken eggs by injection into the allantoic cavities of approximately 10^5 plaque-forming units (PFU) in 0.1 ml of phosphate-buffered saline (PBS). After incubation for 18 h at 37°C, the eggs were chilled and the allantoic fluid was collected. This was used as stock virus. NDV stocks were prepared similarly except that infected eggs were incubated for 48 h at 37°C. Infectivity was determined by plaque assay in CEF monolayers, and the hemagglutinin (HA) was titrated as previously described (11). Neuraminidase (NA) was assayed by the liberation of *N*-acetyl neuraminic acid from fetuin (37). SFV was grown in CEF cells in suspension (19).

Purification of erythrocytes by centrifugation through 10% Ficoll. A 10% solution of Ficoll (Sigma Chemicals Ltd., Poole, Dorset, England) was prepared in PBS and sterilized by autoclaving. Blood cells (2 ml) in Alsever solution were diluted with ice-cold 10% Ficoll (1 ml) to a final concentration of 4×10^8 cells/ml. This mixture (1.5 ml) was layered onto 10 ml of 10% Ficoll and centrifuged at 100 × *g* for 10 min at 4°C. Erythrocytes sedimented to the bottom of the tube leaving a mixture of leukocytes and erythrocytes at the interface (see Results).

Infection of cells. Both CEF cell monolayers and the purified suspension of erythrocytes were infected at a multiplicity of 30 PFU/cell. Virus was adsorbed to cells for 1 h at 37°C. As expected, this caused erythrocytes to aggregate and, where necessary, aggregates were dispersed, as described in Results. Cells were incubated in medium 199 containing 5% newborn calf serum (Flow Laboratories Ltd., Irvine, Scotland) buffered to pH 7.4 with 20 mM HEPES (*N*-2-hydroxyethyl piperazine-*N*'-2-ethanesulfonic acid)-

NaOH. Erythrocytes were kept in suspension on a Rolamix mixing wheel (Luckham & Co. Ltd., Burgess Hill, Sussex, England). Zero time of infection is taken as the time of addition of virus.

Radiolabeling of cells. Before labeling, cells were washed twice with PBS. Labeling was carried out in Earles saline buffered with 20 mM HEPES-NaOH to pH 7.4 and containing 100 μCi of [^{35}S]methionine (specific activity, 800 μCi/mmol, Radiochemical Centre, Amersham) per ml.

PAGE. Gradient slab gels for polyacrylamide gel electrophoresis (PAGE) containing sodium dodecyl sulfate (SDS) (20 by 20 cm) were prepared by using the tris(hydroxymethyl)aminomethane (Tris)-glycine buffer system (23). The polyacrylamide gradient was from 10 to 30% (wt/vol) and contained 0 to 8% glycerol. The ratio of bisacrylamide (Eastman-Kodak, Rochester, N.Y.) to acrylamide was 1:212.

Samples were prepared by the addition of 1/10 of a volume of 1% β-mercaptoethanol together with 1.4% (wt/vol) SDS followed by heating in a boiling water bath for 2 min. After electrophoresis at 20 mA for 13 h the gels were dried under suction onto a filter paper and exposed to Kodirex film (Kodak Ltd., Hemel Hempstead, Herts, England) for 5 to 8 days.

Fluorescent antibody staining. After infection with FP/R for 1 h at 37°C, cells were pelleted and suspended in Earles saline containing 25 mM Tris-hydrochloride (pH 7.7) and 10 mg of trypsin (type III, Sigma Chemicals Ltd., Poole, Dorset, England) per ml to disperse aggregates. Incubation was continued for 30 min at 37°C and the cells were rinsed and resuspended in PBS adjusted to pH 3 for 1 min at 4°C to inactivate residual infectious virus (36). This treatment did not affect the ability of the erythrocytes to synthesize labeled viral proteins as determined by PAGE. After the required periods of incubation at 37°C a smear of infected and noninfected cells in 20% calf serum was made on microscope slides. These were dried with a fan and subsequently fixed in acetone at 4°C. Cells were stained by the indirect technique, firstly with anti-NP serum (18) for 30 min at 37°C and then with fluorescein isothiocyanate-conjugated sheep anti-rabbit immunoglobulin G (Wellcome Reagents Ltd., Beckenham, England) for the same period. After incubation with serum, preparations were soaked in PBS for 40 min with six changes of solution to reduce the background level of fluorescence to a minimum. Fluorescence was observed with a Reichart Binolux II microscope, and all photographic procedures were kept constant to allow direct comparison of the prints.

RESULTS

Preparation of purified erythrocytes. Preliminary experiments showed that newly synthesized radiolabeled viral protein could be detected in washed but unfractionated chicken blood cells infected with FP/R. However, 13-day chicken embryo blood contains a small proportion (0.01%) of leukocytes (35) and it was necessary to separate erythrocytes and leukocytes to show which were responsible for viral protein synthesis.

Cells were separated as outlined in Materials and Methods. The leukocyte fraction was further processed by a second centrifugation through 10% Ficoll. In this way, two populations of cells were obtained: purified erythrocytes and an enriched population of leukocytes in which there was an approximately equal proportion of leukocytes to erythrocytes (i.e., a 10^4-fold enrichment of leukocytes). Blood from 60 embryos yielded approximately 4×10^6 leukocytes. The purity of these cell populations was monitored by phase-contrast microscopy and by the use of a Coulter Channelyzer C-1000 (Coulter Electronics Ltd., Harpenden, Herts, England) which measures the distribution of suspended particles as a function of their volume. Figure 1 is a trace obtained from the Coulter Channelyzer showing that erythrocytes and leukocytes from the interface of the Ficoll separation consist of two size classes. By applying the appropriate formula (16) to the relative volumes, the actual volumes were calculated as approximately 79 and 84 μm^3, respectively. Cells which pelleted through the 10% Ficoll were distributed in a single peak corresponding to a volume of 84 μm^3. These data are consistent with the microscopic observation showing that interface material consists of leukocytes and erythrocytes, whereas pelleted material comprised only erythrocytes. The volumes

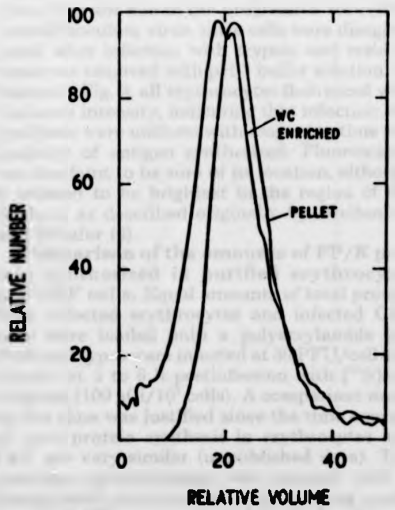

FIG. 1. *Size distribution of purified erythrocytes and of a preparation enriched for leukocytes (WC) made by centrifugation in 10% Ficoll, in which cell volume is plotted against relative cell number. The graph is the direct trace from a Coulter Channelyzer.*

given above are in agreement with values calculated from published figures (35). We conclude that centrifugation through 10% Ficoll offers a procedure for purifying chicken erythrocytes.

Infection of erythrocytes and leukocytes with FP/R. A total of 10^7 unfractionated blood cells, 10^7 purified erythrocytes, and 2×10^6 cells from the enriched leukocyte population were infected with FP/R. Incorporation of trichloroacetic acid-precipitable radioactivity into blood cells or purified erythrocytes was not stimulated on infection. However, newly synthesized radiolabeled viral proteins were detected on analysis by PAGE; Fig. 2 shows that both preparations synthesize viral proteins in approximately equal amounts. No protein synthesis was detected in leukocytes on PAGE even on exposure of the autoradiogram for 8 weeks. Leukocyte viability was confirmed by the exclusion of trypan blue by around 50% of the population. Since the enriched white cell population contained about 10^4-fold more leukocytes than whole blood we conclude that leukocytes are not contributing significantly to the synthesis of viral proteins observed in infected blood. Thus, although it was not necessary to purify erythrocytes, we continued to use the procedure as an added precaution.

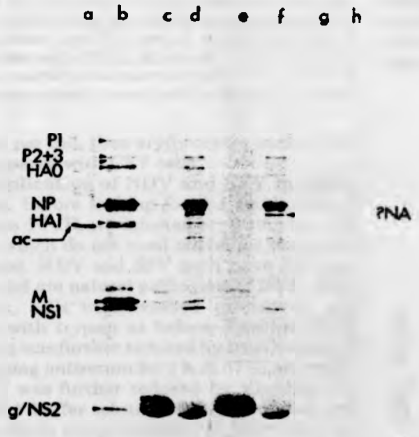

FIG. 2. *PAGE of proteins synthesized in chicken cells infected with FP/R: (a), (b) CEF cells; (c), (d) unfractionated blood cells (10^7/ml); (e), (f) purified erythrocytes (10^7/ml); (g), (h) enriched white cells (2×10^6/ml). (a), (c), (e), and (g) were not infected and (b), (d), (f), and (h) were infected with 30 PFU per cell. Cells were labeled with [^{35}S]methionine from 5 to 6 h postinfection. Host proteins actin (ac) and globin (g) are also indicated. Nomenclature of FP/R proteins follows the established convention (15, 21, 34). Tracks c to f were loaded with approximately equal radioactivity (80,000 cpm) and g and h each were loaded with 8,000 cpm, the maximum available.*

By comparison with the proteins known to be synthesized in CEF cells it is apparent that erythrocytes synthesize P_1, P_2, and P_3, the uncleaved HA precursor which may or may not be glycosylated, NP, M, and NS_1. HA_1 but not HA_2 was detected; in addition, there was a protein migrating in advance of NP which appeared in neither noninfected erythrocytes or CEF cells. This protein is in the position expected of nonglycosylated NA, which in its glycosylated form co-migrates with NP. The smallest viral protein NS_2 was obscured by globin in infected erythrocytes and blood.

Fluorescent antibody staining of FP/R-infected erythrocytes. It was important to determine whether viral proteins were synthesized in all or possibly in only a few particularly productive erythrocytes. Infected erythrocytes stained with anti-NP serum fluoresced more brightly than noninfected cells. However, the level of fluorescence was low at all times. Positive fluorescence was found at 3 h postinfection and this increased up to 8 h postinfection (Fig. 3) showing that NP antigen is synthesized de novo. It is not due to the progressive accumulation of inoculum virus, since cells were disaggregated after infection with trypsin and residual virus was removed with pH 3 buffer solution. As shown in Fig. 3, all erythrocytes fluoresced with the same intensity, indicating that infection and synthesis were uniform with respect to time and quantity of antigen synthesized. Fluorescence was too faint to be sure of its location, although it seemed to be brightest in the region of the nucleus, as described originally by Breitenfeld and Schafer (6).

Comparison of the amounts of FP/R protein synthesized in purified erythrocytes and CEF cells. Equal amounts of total protein from infected erythrocytes and infected CEF cells were loaded onto a polyacrylamide gel. Both cell types were infected at 30 PFU/cell and labeled at 5 to 6 h postinfection with [^{35}S]methionine (100 μCi/10^7 cells). A comparison made at this time was justified since the time courses of viral protein synthesis in erythrocytes and CEF are very similar (unpublished data). The resulting autoradiogram was scanned with a Joyce-Loebl densitometer, and the area under the viral NP peak produced by each cell type was calculated. When normalized for total protein, erythrocytes synthesized 5% of the amount of NP found in CEF cells. However, if the calculations are made on the amount of NP syn-

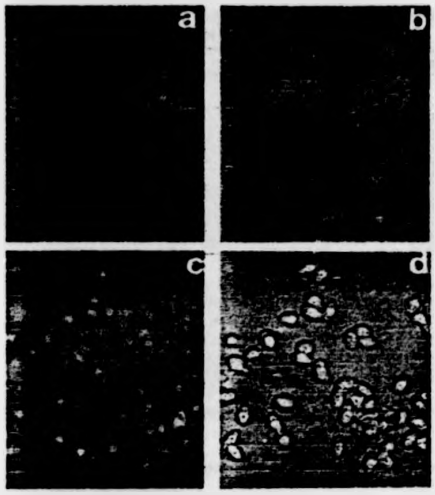

FIG. 3. *Fluorescent antibody staining of (a) noninfected erythrocytes, (b) infected erythrocytes at 3.5 h postinfection, and (c) infected erythrocytes at 8 h postinfection with FP/R; (d) shows the same field as (c) viewed under phase-contrast optics. All aspects of photography of the stained cells were kept constant.*

thesized per cell, then erythrocytes make 0.65% NP compared with CEF cells.

Multiplication of NDV and SFV in erythrocytes. Before looking for the production of infectious FP/R, we infected erythrocytes with viruses which do not need nuclei for their multiplication. NDV and SFV both have RNA genomes and are natural pathogens of birds. After infection, cells were washed extensively and treated with trypsin as before. Residual NDV inoculum was further reduced by treatment with neutralizing antiserum for 1 h at 37°C and residual SFV was further reduced by washing cells with pH 3 buffer solution. Samples of cells and culture fluids were disrupted at intervals after infection, and infectivity was measured by plaque assay on CEF monolayers. There was an increase in infectivity of about 100-fold of NDV and SFV during the incubation time (Fig. 4), showing that erythrocytes are able to sustain a productive infection by both viruses.

Failure of FP/R to multiply in erythrocytes. Erythrocytes were infected as described previously, and after 1 h at 37°C, cells were washed, disaggregated with trypsin, and treated with pH 3 buffer solution as described above.

Cells were disrupted in culture fluids at intervals after infection and assayed on CEF monolayers. No rise in the amount of infectious virus present was detected (Fig. 4). Titrations were repeated in suspension plaque assays (26) in the presence of trypsin to enhance infectivity (1, 10). Again the level of infectious virus present did not rise above the initial level of 900 PFU/10^7 cells even though A/PR/8/34, which requires trypsin for plaquing, was assayed successfully (Table 1). By calculating the ratio of NP synthesized in CEF cells to NP synthesized in erythrocytes, and by knowing the number of plaque-forming units synthesized in CEF cells, we estimated that the infectivity in erythrocytes was over 400-fold less than the expected value (Table 2). The expected-observed ratios for HA and NA are discussed in the next section.

Hemagglutination by and NA activity in FP/R-infected erythrocytes. Erythrocytes were infected, and residual inoculum was reduced as described for the preparation of cells for fluorescent antibody staining. Samples of cells were removed at intervals and disrupted by ultrasonication on ice. No hemagglutinating activity was detected. Standard NA assays incubated for 1 h at 37°C failed to give a positive result so incubation was continued for 30 h. This revealed NA activity which increased with the duration of infection (Fig. 5) and which was

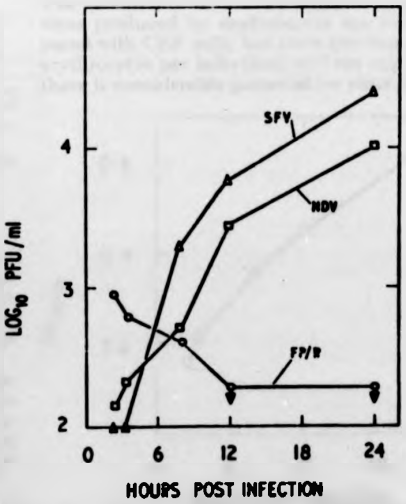

FIG. 4. *Extent of multiplication in erythrocytes of NDV, SFV, and FP/R. Cells (10^7/ml) were infected at 30 PFU/cell and then treated to remove inoculum as described in Materials and Methods. Samples were taken at intervals, and production of infectious virus was measured by plaque assay.*

TABLE 1. *Cell suspension plaque assay for the multiplication of FP/R in erythrocytes*[a]

Virus	+ Trypsin	− Trypsin
FP/R in erythrocytes postinfection (hours)		
2.5	900[b]	NT[c]
3.5	600	NT
8.0	400	NT
12.0	<150	NT
24.0	<150	<150
A/FP/Rostock[d]	8.9×10^8	8.0×10^4
A/PR/8/34[d]	3.1×10^7	$<10^2$

[a] Samples of erythrocytes were mixed with 2.5 ml of 3×10^7 CEF cells in suspension and dispersed in an equal volume of double-strength overlay medium containing 20 μg of diethylaminoethyl-dextran and 100 μg of trypsin per ml. FP/R and PR/8 were inoculated directly into the CEF suspension.

[b] Number of plaque-forming units per 10^7 erythrocytes.

[c] NT, Not tested.

[d] Number of plaque-forming units per milliliter of virus suspension.

TABLE 2. *Synthesis of PFU, HA, and NA activities in FP/R-infected erythrocytes*[a]

Cells infected	PFU	NA	HA
Erythrocytes			
Expected	6×10^4	0.11	13.0
Observed	1.5×10^2	0.14[b]	<0.6
Expected-observed	400	0.8	≥21.7
CEF	8.5×10^{6c}	17.0[d]	2,000

[a] Expected values were calculated from the ratio of ND synthesized in CEF cells and erythrocytes, i.e., 100:0.65 arbitrary units (see text).

[b] Optical density at 549 nm per hour calculated from a 30-h incubation.

[c] All figures are for 10^7 cells plus culture fluids.

[d] Optical density at 549 nm per hour.

serologically identical with the FP/R virion enzyme (data not shown). It was calculated that the ratio of NP to NA activity synthesized in erythrocytes is similar to that in CEF cells (Table 2). However, the relative HA titer was more than 6.5-fold lower than expected.

Viral proteins synthesized by erythrocytes infected with NDV and SFV. Since these viruses multiplied in erythrocytes we investigated the synthesis of viral proteins. Figure 6 shows PAGE autoradiograms of cells infected by the standard procedure with NDV or SFV. In the former there was very little detectable viral protein synthesis and in the latter there was none (compare with FP/R in Fig. 2), even at times when infectious virus production had increased by 100-fold. The major NDV polypeptide appeared in the 55,000 to 65,000 molecular weight region, which agrees with a previous study (8). In another experiment, a faint band was seen in SFV-infected erythrocytes (data not shown), corresponding to the envelope protein.

DISCUSSION

We have shown that purified erythrocytes can be infected with FP/R and will synthesize viral proteins. Confirmation that leukocytes were not responsible for viral synthesis was obtained when a leukocyte preparation containing approximately 10^4-fold more leukocytes than unfractionated blood failed to synthesize any detectable viral proteins. The question of whether all erythrocytes were equally capable of being infected or whether the observed viral protein was synthesized by a few highly productive erythrocytes was resolved by fluorescent antibody staining; NP antigen was detected at a uniform level in all erythrocytes.

To our knowledge this is the first account of de novo infection and synthesis of viral components in erythrocytes. However, no infectious FP/R was produced by the infected erythrocytes. This is not due to an inability of these cells to form progeny virus particles per se since two other enveloped viruses (NDV and SFV) were able to productively infect erythrocytes. The amounts of viral components or infectious virus produced by erythrocytes are low compared with CEF cells, but since the numbers of erythrocytes per individual bird are very large, there is considerable potential for virus produc-

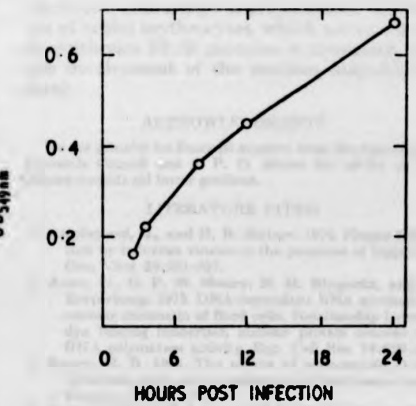

FIG. 5. *Synthesis of active NA in FP/R-infected erythrocytes. Samples of 10^7 erythrocytes were assayed for the presence of NA by incubation with fetuin for 30 h at 37°C. The initial value is thought to represent residual inoculum.*

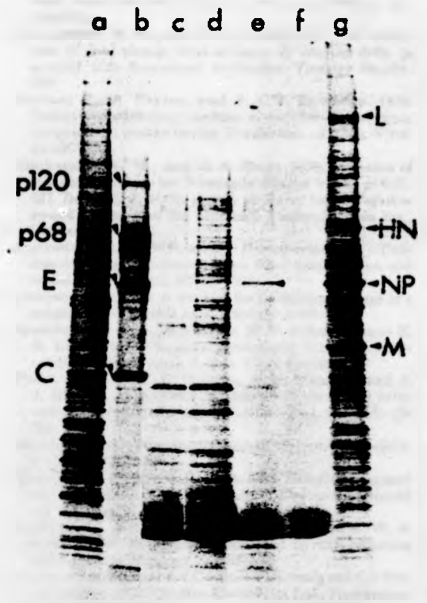

FIG. 6. *PAGE of proteins synthesized by erythrocytes and CEF cells infected with NDV or SFV under the same conditions as those used in Fig. 2 for FP/R. Viral proteins are arrowed. (a) Noninfected CEF cells; (b) SFV-infected CEF cells both a and b pulsed with [^{35}S]methionine from 6 to 6.5 h postinfection; (c) SFV-infected erythrocytes pulsed from 9 to 9.5 h postinfection; (d) noninfected erythrocytes; (e) (f) NDV-infected erythrocytes pulsed 3 to 3.5 and 9 to 9.5 h postinfection respectively; (g) NDV-infected CEF cells. The experiment with (c), (e), and (f) was done in the presence of 3 μg of actinomycin D per ml to reduce endogenous protein synthesis and enhance the detection of viral proteins. Nomenclature of SFV and NDV proteins follows the conventions (7, 8).*

tion. However, we have no experimental knowledge of the significance of our findings to pathogenesis.

The production of infectious progeny virus was not related to the amount of newly synthesized viral protein accumulating inside the cell, since the proteins specified by SFV and NDV were barely detectable, whereas the FP/R-directed synthesis was considerable. The reason why no FP/R progeny virions were formed did not emerge. We eliminated the possibility that it was due to the failure of HA_0 to be cleaved, by plaquing in the presence of trypsin which cleaves HA_0 and activates infectivity (22, 25). Another reason for this lack of infectious FP/R could be associated with the levels of functional HA found in infected cells (Table 2). Since the relative amount of HA_0 polypeptide was normal, the absence of functional protein may be due to a defect in posttranslational modification (evidenced perhaps by the absence of HA_2), although the erythrocytes evidently carry out analogous processing on NDV and SFV. However, there are many other possible defects in virion formation which might account for the lack of production of infectious virus observed. Even so, we have not ruled out that erythrocytes are making very low levels of infectious virus. This possibility is relevant to the "recycling" hypothesis of antigenic shift in the antigenicity of influenza viruses, which proposes that strains lie dormant and reemerge later to infect humans when herd immunity falls to an ineffective level (20). As yet no source of persistent or chronic infection of influenza has been identified.

While this work was in progress a parallel arose between the FP/R-erythrocyte system and the coupled transcription-translation in vitro system (9, 31). The erythrocyte nucleus is dormant (compared with a normal dividing cell) and the in vitro system does not require nuclei for the synthesis of viral proteins. This possibility was tested by the use of actinomycin D, which does not inhibit the in vitro system (31), but which abolished the synthesis of FP/R proteins in erythrocytes, showing that nuclear function(s) were indeed operating (Cook, Avery, and Dimmock, manuscript in preparation). The failure of rabbit erythrocytes, which are enucleate, to synthesize FP/R proteins is consistent with this involvement of the nucleus (unpublished data).

ACKNOWLEDGMENTS

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DIFFERENTIAL DISTRIBUTION OF INFLUENZA VIRUS P PROTEINS IN NUCLEI OF INFECTED CELLS

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Received 27 June 1979

1. Introduction

The genome of avian influenza type A viruses comprises eight segments of vRNA [1–4] which are complementary to viral mRNA [5,6]. The latter are monocistronic and code for the following proteins in descending order of MW: P1, P2, P3, HA, NP, NA, M and NS_1 [7–10]. NS_1 and an additional virus specified protein NS_2 are found only in infected cells [11–13]. The other proteins are constituents of the virion.

P1, P2 and P3 are located together with NP and vRNA inside the virion in an RNP complex which will synthesise complementary RNA in vitro [8,14,15]. Genetic evidence confirms that the P proteins are concerned with transcription and further that they have a role in replication (see reviews [16,23]). The precise functions of P1-3 are unknown although a mutant, defective in the P2 gene (coding for the P1 protein [21]) failed to transport viral RNA from the nucleus [16].

Influenza virus multiplication is absolutely dependent upon the cell nucleus, requiring initially DNA-dependent RNA polymerase II activity [17,18] to synthesise functional viral message [19]. Later in infection, newly synthesised v and mRNA [19–21] and NP, M and NS_1 proteins are found in the nucleus [22,23]. The various functions of these components in the nucleus is unknown, although it is suggested that vRNA synthesis occurs in that location [21,24] and that assembly of vRNA and proteins destined for progeny virions takes place there [23].

Identification of the location of P proteins in infected cells is difficult because they are synthesised in low amounts. However, their involvement in viral RNA synthesis stimulates investigation and we report here on the transfer of newly synthesised P1, P2 and P3 proteins to the nuclei of infected cells.

2. Materials and Methods

2.1. Cells and virus

Chick embryo fibroblast monolayers were infected with avian influenza virus A/FPV/Rostock/34 (Hav1N1) as described before [25]. Nuclei were obtained by the "nuclear monolayer method" [26] in which the cell monolayer is washed with non-ionic detergent (2% NP40 in PBS at 4°C). This treatment leaves nuclei in situ and they are then scraped off and further purified by washing in 0.25 M sucrose, 0.01 M Tris pH 7.4, 1 mM $MgCl_2$.

2.2. Radiolabelling and analysis of proteins

Before adding radiolabel, cells were rinsed and put into Earle's saline with 4% foetal calf serum (dialysed against saline). 10 min later, cells were pulsed for 15 min with 1 ml saline containing 100 μCi [^{35}S]-methionine (spec. act. 800 Ci/mmol; Radiochemical Centre, Amersham). Monolayers were rinsed three times with medium 199 containing 5% newborn calf serum and 7.5 μg/ml unlabelled methionine and incubation was continued in the same medium. Proteins were analysed by polyacrylamide gel electrophoresis on a linear gradient of acrylamide (10–30% w/v [27] and the dried gel was autoradiographed.

3. Results and Discussion

Fig. 1(a) shows the overall distribution of viral proteins in nuclei at various times after labelling

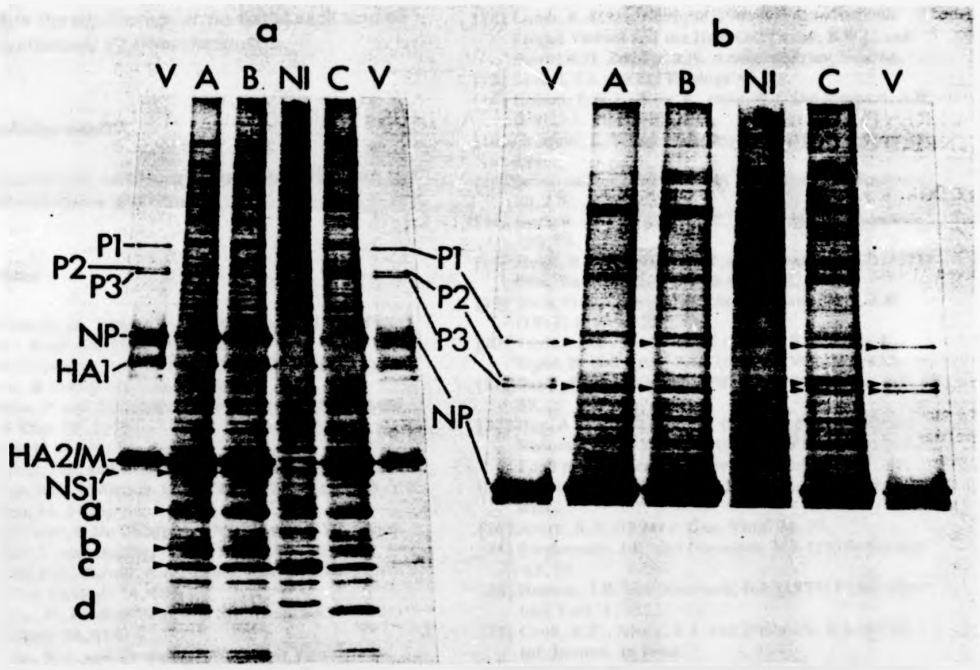

Fig. 1.(a) Polyacrylamide gel electrophoresis of proteins from nuclei of CEF cells infected with A/FPV/Rostock/34 and radiolabelled for 10 min with [^{35}S]methionine at 4 h p.i. (A); incubated for an additional 10 min in the absence of radiolabel (B); and for an additional 2 h in the absence of radiolabel (C). Non-infected cells (NI) were labelled for 10 min in parallel with (A). Marker radiolabelled virions (V) were also analysed. (b) The right hand panel shows an enlargement of the region containing the P proteins.

together with a [^{35}S]methionine-labelled virion marker [28] which clearly shows the separated P1, P2 and P3 proteins. Nuclei isolated immediately after the pulse contained large amounts of NP, M and NS_1 as previously reported [23]. Also present in nuclei are at least four proteins migrating ahead of NS_1 (labelled a–c) which are similar in size to those reported by others [12,13]. A photographic enlargement of the region of the gel resolving the high mol. wt. proteins was made to more easily distinguish between viral and cellular proteins (Fig. 1b). After pulsing at 4 h p.i. for 10 min, P1, P2 and P3 were present in approximately equal amounts in whole cell extracts (data not shown) but isolated nuclei contained only P1 and P3. Thus at this stage P2 was confined to the cytoplasm. There was no change in distribution of the P proteins after an additional 10 min incubation in unlabelled medium but after 2 h incubation (to 6 h p.i.) P2 was present in nuclei in about the same amounts as P1 and P3.

This report shows that P2 behaves differently from P1 and P3 in regard to its transfer to the cell nucleus. In conjunction with genetic studies [21], which show that virus with a mutation in P3 is deficient in vRNA but not cRNA synthesis, our data showing rapid transfer of P3 into the nucleus are consistent with the suggestion that rRNA is synthesised in the nucleus. Further studies are in progress to

investigate the significance of the initial exclusion of newly synthesised P2 from the nucleus.

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Archives of Virology

Complementation with an Avian Influenza Virus is Required for Synthesis of M Protein of a Human Strain in Chicken Erythrocytes

Brief Report

By

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With 4 Figures

Accepted April 3, 1980

Summary

The M protein of avian, but not human, strains of influenza A viruses is synthesized in infected chicken erythrocytes. In dual infections an avian strain complemented the human virus and both the human and avian M proteins were expressed.

*

We recently reported that purified chicken erythrocytes could be infected with an avian strain of type A influenza virus (4). No infectious progeny were detected but virus proteins were synthesized to about 0.7 per cent of the amount made in a chick embryo fibroblast (CEF) cell. The relative proportion of proteins was similar in both cell types. In this report we describe the ability of the chicken erythrocytes to support the synthesis of human type A influenza virus proteins.

Erythrocytes were obtained from 13 day chicken embryos of a hybrid strain derived from a Light Sussex female and a CO20 male. Cells were purified by sedimentation through Ficoll (Sigma Chemicals Ltd., Poole, Dorset) (4). No white cells could be detected in the purified preparation. All other experimental procedures involving infection, radiolabelling and analysis of virus proteins by polyacrylamide gel electrophoresis (PAGE) were as previously described (4).

The synthesis of virus proteins in erythrocytes infected with A/WSN (H0N1) is shown in Fig. 1. Although NP and NS1 were clearly present from 3.5 hours p.i. no M was detected even at 9.5 hours p.i. No M protein was detected in similar experiments with other human strains including A/NWS, A/PR/8/34 (H0N1), A/Japan/305/57 (H2N2) and A/HK/1/68 (H3N2). These results in Fig. 1 can be compared with a time course of the synthesis of proteins directed by the avian strain A/FPV/Rostock/34 (Hav1N1) (Fig. 2). P1, P2, P3, NP and NS1 were all

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detectable at 2.5 hours post infection (p.i.) while M was present in substantial amounts at 3.5 hours p.i.

In avian virus-infected erythrocytes there was an unidentified protein, slightly smaller than HA (Fig. 2) which comigrated with a cellular protein. Unlike cellular proteins such as actin, its synthesis increased and was maintained during infection. It appears to be either a privileged cellular protein or an unknown viral protein. Both the kinetics of synthesis of FP/Rostock proteins and the shut-off of cell

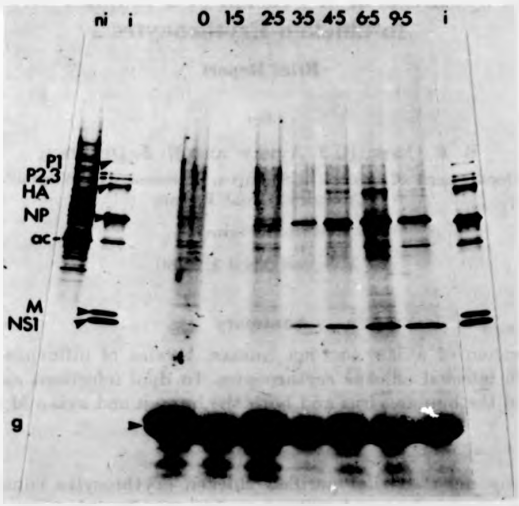

Fig. 1. PAGE of protein synthesis by A/WSN in erythrocytes from 13 day old chick embryos. Infected *(i)* nad non-infected *(ni)* markers of CEF cells are included. Cells were infected with 30 PFU/cell and labelled with 100 μCi/ml/10^7 cells ^{35}S-methionine for 30 minutes. Erythrocytes were harvested at the times indicated in hours p.i. *ac* actin, *g* globin

protein synthesis in erythrocytes were similar to those found earlier in CEF cells (15) indicating that in this regard virus expression was unaffected by the unusual structural and metabolic features of erythrocytes. This was surprising as the highly differentiated and non-dividing state of avian erythrocytes (6, 10, 16, 17, 22), lack of endoplasmic reticulum, presence of few ribosomes and mitochondria (6, 18, 21, 2) and a nucleus which synthesizes little, if any, DNA and only trace amounts of RNA (1, 7, 9, 11, 20, 22) might have been expected to set them apart from relatively non-differentiated dividing cells.

The inability of certain cell lines to synthesize M protein has been noted by others (3, 8, 19) but this is the first instance of the defect being virus strain specific. Since FP/Rostock synthesized M while the human influenza strains did not, we set

up a "complementation" experiment in which cells were dually infected with both a human and an avian strain. We chose A/FPV/Dutch/27 (Hav 1 Neq 1) and A/NWS as their M proteins migrated at slightly different rates on PAGE. Fig. 3 shows that in the mixed infection, the M protein of both the avian strain and the human strain were synthesized and were present in comparable amounts. Thus the erythrocytes are not intrinsically deficient in their ability to make human M protein. Apparently expression of M has to be switched on by some virus function which the human strain cannot provide in erythrocytes but avian strains can. Thus

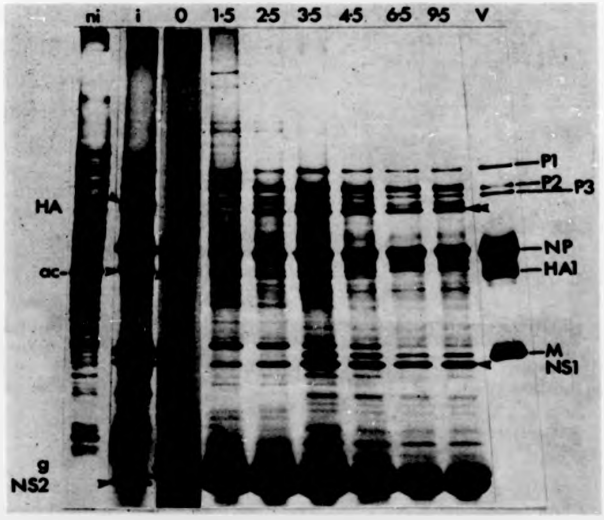

Fig. 2. PAGE of proteins synthesized in FP-R/infected erythrocytes. Infected *(i)* and non-infected *(ni)* CEF cells and ^{35}S-methionine-labelled virions *(V)* (6) are included as markers. Experimental conditions are described in Fig. 1. An unidentified protein which increases after infection is arrowed (◂◂)

we conclude that expression of M is an event which is directly or indirectly consequent upon some earlier viral function. We have no information as to its nature but there is evidence that a host cell function is involved since differential inhibition of M has been observed in cells treated with graded doses of actinomycin D(AMD) or camptothecin (13) or irradiated with UV-light prior to infection (12, 14). Fig. 4 shows that in infected erythrocytes treated with AMD at concentrations of 0.1 to 0.3 μg/ml M protein synthesis is particularly sensitive to inhibition.

Thus we have a situation where cellular variation (3, 8, 19), variation of virus strains (this report) or the action of inhibitors (12—14) can differentially inhibit the expression of the M protein. We do not as yet know why only the M protein is affected. or what the underlying mechanisms of this effect are.

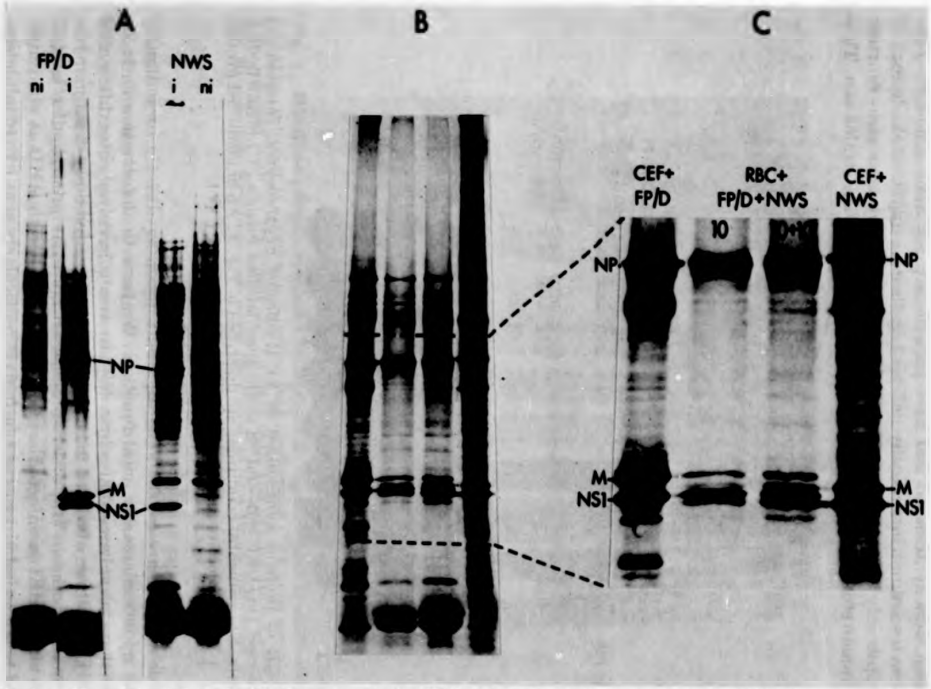

Fig. 3. Synthesis of M proteins infected with the avian strain FP/Dutch and the human strain A/NWS. Experimental conditions are described in Fig. 1. *A* Erythrocytes infected *(i)* or non-infected *(ni)* with FP/Dutch or A/NWS (about 10 and 100 PFU/cell respectively) and labelled from 4 to 4.5 p.i. *B* Mixed infection experiment of which part of the gel is photographically enlarged *(C)*. Outer tracks: infected CEF markers; inner tracks: mixed infection of erythrocytes pulsed for 10 minutes or pulsed and chased for an additional 10 minutes (10 + 10) at 4 hours p.i.

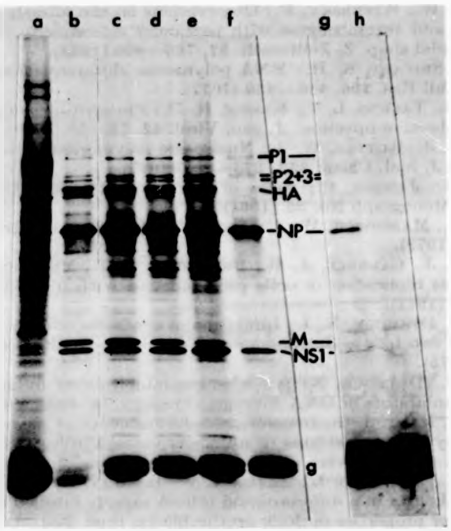

Fig. 4. Inhibition of synthesis of FP/R proteins in erythrocytes pretreated with various concentrations of AMD for 1 hour before and throughout infection. Cells were labelled from 5 to 6 hours p.i. as described in Fig. 1. *a* non-infected, *b* infected cells without AMD; with AMD at 0.01 *(c)*, 0.03 *(d)*, 0.1 *(e)*, 0.3 *(f)*, 1.0 *(g)* and 3.0 μg/ml *(h)*. *a* to *f* and *g*, *h* are from separate experiments

Fig. 3. Synthesis of M proteins infected with the avian strain FP/Dutch and the human strain A/NWS. Experimental conditions are described in Fig. 1. *A* Erythrocytes infected *(i)* or non-infected *(ni)* with FP/Dutch or A/NWS (about 10 and 100 PFU/cell respectively) and labelled from 4 to 4.5 p.i. *B* Mixed infection experiment of which part of the gel is photographically enlarged *(C)*. Outer tracks: infected CEF markers; inner tracks: mixed infection of erythrocytes pulsed for 10 minutes or pulsed and chased for an additional 10 minutes (10 + 10) at 4 hours p.i.

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